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La función metabólica de la AOX y NDH-2 en *Ustilago maydis*

T E S I S

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ECCE LUPUS STIPPARIUM

Teatro mágico.
Entrada no para cualquiera.
No para cualquiera...
Sólo para locos.
La entrada cuesta la razón.
Hermann Hesse, 1928.

Amo al que se hunde, pues es quien
cruza el abismo...
Amo al que tiene espíritu libre y
corazón libre, pues su mente no es sino
la entraña de su corazón, mas su
corazón lo hunde.
Friedrick Nietzche, 1888.

DEDICATORIAS

Para Germán, a quién sólo puedo ofrecer el mejor mal ejemplo, y todo mi maldito y oscuro corazón.

"Coatlicue era la Tierra, madre de Coyolxauhqui, la Luna, y de los Cuatrocientos del sur (los inumerables) Centzon Huiznahua, las Estrellas. Un día, cuando barría su templo en lo alto del cerro de Coatepec, Coatlicue quedó embarazada milagrosamente gracias a una bolita de plumas que provenía del cielo y que ella guardó en su seno. Coyolxauhqui consideró el embarazo de su madre como una afrenta e instigó a sus hermanos las Estrellas a matarla. Huitzilopochtli, el Sol, desde el vientre de la Tierra, advirtió el peligro y decidió defender su vida y la de su madre. Cuando la Luna y las Estrellas estaban a punto de asesinar a Coatlicue, nació Huitzilopochtli, ataviado para la guerra y armado con una serpiente de fuego, llamada Xiuhcoatl, con la que la decapitó a la Luna para después, arrojarla desde lo alto del cerro de Coatepec. En su caída, la diosa se fue despedazando en cada giro, para yacer finalmente desmembrada al pie del cerro. Huitzilopochtli persigió a sus hermanos hasta los confines del mundo y también terminó con ellos.
Así muere la Luna cada mes derrotada por el Sol, a pedazos (en fases). Así el Sol se abre paso cada día de entre las tinieblas, armado con el rayo de luz -Xiuhcoatl-, desterrando a la Luna y a las Estrellas del espacio celeste, para que al final del día caiga ensangrentado –cubierto de rojo– en los brazos de su madre, la Tierra".

Leyenda Nahua

Para Karina.

"Lay beside me, tell me what have they done
Speak the words I wanna hear, to make my demons run...
Lay beside me, under wicked sky
The black of day, dark of night, we share this paralyze
The door cracks open, but there's no sun shining through
Black heart scarring darker still, but there's no sun shining through

What I've felt, what I've known
Turn the pages, turn the stone
Behind the door, should I open it for you?
What I've felt, what I've known
Sick and tired, I stand alone
Could you be there, 'cause I'm the one who waits for you
Or are you unforgiven too?

Come lay beside me, this won't hurt I swear
She loves me not, she loves me still, but she'll never love again
She lay beside me, but she'll be there when I'm gone
Black hearts scarring darker still, yes she'll be there when I'm gone
Yes she'll be there when I'm gone
Dead sure she'll be there

Lay beside me, tell me what I've done
The door is closed, so are your eyes
But now I see the sun, now I see the sun
Yes, now I see it"

Metallica, 1997

Para mi Mamá, para mi Abuelita y para mis Hermanas.

<div align="right">

"Is this the real life?
Is this just fantasy?
Caught in a landslide
No escape from reality
Open your eyes
Look up to the skies and see

I'm just a poor boy, I need no sympathy
Because I'm easy come, easy go,
A little high, little low,
Anyway the wind blows, doesn't really matter to me,
to me

Mama, just killed a man
Put a gun against his head
Pulled my trigger, now he's dead
Mama, life had just begun
But now I've gone and thrown it all away
Mama didn't mean to make you cry
If I'm not back again this time tomorrow
Carry on, carry on, as if nothing really matters"
Queen, 1975

</div>

Para mis hermanos, no de sangre, sino de corazón, Irvin Garduño, Ismael Ramírez, Julián Almazán y Marcos Vázquez.

Para mis viejitos, Sidonio, Eloisa, Elpidio y Quintín.

Para mi familia Mendoza y mi Familia Tuz Moya.

Para mis hermanos mudos.

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A Germán, a Karina, a mi familia y amigos, por que nada tendría sentido y nada hubiera sido sin ustedes.

> *"¿Qué sería de ti poderoso astro rey, sino tuvieras aquellos para los que brillas?"*
> Friedrick Nietzche, 1888.

Al Dr. Juan Pablo Pardo, por todas las facilidades con las que conté en su laboratorio para realizar el trabajo experimental. Pero sobretodo agradezco las conversaciones, risas, discusiones, y la huella profunda e indeleble que imprimió en mí en estos pocos años.

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A la Facultad de Ciencias, a la Facultad de Medicina y a la UNAM, por sus entrañables espacios y por darme la oportunidad de convertirme en lo que decidí ser.

A los hombres y mujeres que a través de sus palabras, sus estrofas, sus ideas o sus acciones han influido en mí, por que sus voces retumban fuerte en mis oídos.

Índice

RESUMEN

Ustilago maydis es un hongo fitopatógeno que produce la enfermedad denominada carbón del maíz, la cual causa pérdidas económicas importantes. Por esta razón se ha estudiado ampliamente, de tal forma que actualmente se considera un organismo patógeno modelo. Sin embargo, diversos aspectos de su biología no se encuentran descritos, particularmente su metabolismo energético e intermediario. En este trabajo se abordó el estudio de la organización de la cadena respiratoria mitocondrial de *U. maydis* y del papel metabólico de la isoforma externa de la NADH dehidrogenasa alterna (ND2e) y de la oxidasa alterna (AOX).

a) **La organización de la cadena respiratoria.** La mitocondria de *U. maydis* presenta los cuatro complejos clásicos de la cadena transportadora de electrones (complejos I, II, III y IV), junto con con la glicerol 3- fosfato deshidrogenasa mitocondrial y dos componentes respiratorios accesorios, la ND2e y la AOX.

La ND2e cataliza la transferencia de los electrones del NADH a la ubiquinona. En contraste con el complejo I no bombea protones a través de la membrana interna mitocondrial, está compuesta por un sólo péptido y por un cofactor, y es insensible a la rotenona. Esta enzima presenta una actividad substancial en *U. maydis*, lo que hace pensar que puede contribuir de manera significativa a la oxidación del NADH citosólico. Contrario a lo reportado para las mitocondrias de plantas o de otros hongos, no se activa en presencia de Ca^{2+}.

La AOX es una oxidasa terminal que cataliza la transferencia de electrones del ubiquinol hacia el oxígeno molecular. En contraste con la vía citocrómica, compuesta por los complejos III y IV, no bombea protones y es resistente al cianuro y a la antimicina, pero se inhibe con agonistas fenólicos, como el ácido salicilhidroxámico o los alquil galatos. En *U. maydis* la actividad de la AOX fue del 20% con respecto a la respiración original. No obstante, esta actividad muestra dependencia con respecto a las condiciones de cultivo, y en las células cultivadas en presencia de cianuro o antimicina puede representar hasta el 75% de la respiración original.

Al estudiar los mecanismos regulatorios de la AOX de *U. maydis* se determinó que esta enzima se activa en presencia de AMP y no responde al piruvato, lo cual concuerda con las propiedades regulatorias de una AOX de los hongos. Sin embargo, el agente reductor DTT, que activa a las AOX de plantas, es capaz de proteger a esta enzima durante el proceso de permeabilización celular.

También se describió que las actividades de las rutas de reducción y oxidación del ubiquinol o ubiquinona no presentan aditividad, lo que indica que los componentes respiratorios compiten entre sí por estos intermediarios membranales.

b) **El papel metabólico de la oxidasa alterna.** En este trabajo se analizó el efecto de algunas condiciones de cultivo que podrían ser consideradas de relevancia ecológica para *U. maydis*, tales como la temperatura de crecimiento, la riqueza del medio de cultivo y la fase de crecimiento, sobre la función mitocondrial, con el objeto de entender el papel fisiológico de la AOX.

Se encontró evidencia de que la AOX tiene dos funciones principales en *U. maydis*: a) forma parte importante de los sistemas de prevención del estrés oxidativo, característica ampliamente descrita en otros organismos, y b) es uno de los mecanismos que aseguran e incrementan la plasticidad metabólica de la célula, permitiendo que el metabolismo celular no se detenga en condiciones donde la vía citocrómica se encuentra limitada. Esta función podría ser valiosa para organismos que están sujetos a variaciones en la temperatura, en la composición del medio o en la fuente y disponibilidad nutrimental, entre otros.

Debido a que la actividad de la AOX puede ser perjudicial para el estado energético de la célula, se encuentra estrictamente regulada, de manera que sólo es activa bajo estados metabólicos particulares. Además, en condiciones regulares tiene una baja participación en la respiración celular.

También se describió que los mecanismos de regulación de la AOX de los hongos contrastan con los de plantas. La activación de la AOX en los hongos se lleva a cabo en estados energéticos bajos, mientras que en las plantas ocurre cuando el estado energético de la célula es alto.

c) **La función metabólica de la NADH deshidrogenasa alterna.** Una de las funciones más importantes de la mitocondria es la de oxidar al NADH que se produce en el citosol. Debido a que la membrana interna mitocondrial es impermeable a esta molécula, se requiere que los electrones sean incorporados a diversos intermediarios carbonados, para posteriormente ser introducidos a la cadena respiratoria. Los mecanismos encargados de este proceso se conocen como lanzaderas y existen una gran variedad de ellas. Ya que *Ustilago maydis* no es un organismo fermentador, es posible que la mayoría del NADH citosólico sea oxidado por la mitocondria, por lo que es importante caracterizar los mecanismos de lanzadera presentes en este hongo. A pesar de que *U. maydis* tiene el complemento genético para todas las lanzaderas reportadas, los datos sugieren que el sistema más importante es la ND2e, debido a que su actividad fue superior que la de las otras lanzaderas y además es constitutiva. Sin embargo, la ND2e puede estar acompañada por la lanzadera de glicerol 3-fosfato, y posiblemente por la de malato-oxaloacetato o la de acetaldehído-etanol, aunque estas actividades dependen de las condiciones particulares de cultivo.

ABSTRACT

Ustilago maydis is a fungal phytopathogen that produces the corn smut disease, which generates large economic losses. For this reason, it has been extensively studied and actually it is considered a model pathogen organism. However, many aspects of its biology remain unstudied, in particular its energetic and intermediary metabolism. In the present work the organization of the mitochondrial respiratory chain of *U. maydis* cells was studied, along with the metabolic roles of the external isoform of the alternative NADH dehydrogenase (ND2e) and the alternative oxidase (AOX).

a) **Respiratory chain organization.** *U. maydis* mitochondria contain the four classical components of the electron transport chain (complexes I, II, III, and IV), the mitochondrial glycerol 3-phosphate dehydrogenase and two alternative elements, the ND2e and the AOX.

The ND2e catalyzes the electron transfer between NADH and ubiquinone. In contrast with complex I, the ND2e does not pump protons across the mitochondrial inner membrane, it is composed by a single polyeptide and by one cofactor, and is insensitive to rotenone. In *U. maydis* this enzyme shows a considerable activity and seems to participate in the metabolic systems involved in cytosolic NADH oxidation. Its activity is not modulated by Ca^{2+}, a regulatory mechanism described for plant and other fungal ND2e.

The AOX is a terminal oxidase that catalizes the electron transfer between the ubiquinol molecule and oxygen. In contrast with the cytochrome pathway, composed by complexes III and IV, the AOX does not pump ions and is resistant to cyanide and antimycin, but is inhibited by phenolic molecules, such as salicylhydroxamic acid and alkyl gallates. In *U. maydis*, the AOX activity accounts for the 20% ot the uninhibited respiratory rate, and seems to depend on growth conditions, as in cells cultured in the presence of cyanide or antimycin, where the activity increase to about 75% of the uninhibited rate.

The study of the regulatory mechanisms of *U. maydis* AOX showed that this enzyme is activated in the presence of AMP, but not with pyruvate, which is consistent with

the regulatory characteristics of a fungal AOX. However, the reducing agent DTT, which activatees the plant AOX, protected the enzyme against inactivation during cell permeabilisation.

The activity of the ubiquinone reduction and ubiquinol oxidation pathways do not show additive behavior, which indicates that the pathways for the ubiquinone reduction and ubiquinol oxidation compete for this membrane intermediates.

b) **The metabolic role of alternative oxidase.** In this work, the effect of some culture conditions, which could be of ecological significance for *U. maydis*, such as the growth temperature, the nutrient richness of the culture media and the growth phase, was evaluated over the mitochondrial function, with the purpose to understand the physiologic role of AOX.

The evidence indicates that AOX accomplish two major functions in *U. maydis*: a) forms part of the oxidative stress handling machinery, a well described issue in other organisms, and b) as part of the mechanisms that increase the metabolic plasticity of the cell, allowing the metabolism to proceed in conditions where the cytochrome pathway is restricted, a role that might be valuable for organisms exposed to variations in temperature, in the medium composition, in the nutrient source and availability.

Since the AOX activity could be detrimental for the energetic state of the cell, it is strictly regulated, and becomes active under particular metabolic states. In adddition, in regular conditions the AOX has a low contribution to cell respiration.

The regulatory mechanisms of the AOX of plants and fungi are contrasting. In fungi, the activation is achieved under low energetic conditions, while in plants the activation is reached under high energetic statuses.

c) **The metabolic role of the alternative NADH dehydrogenase.** One of the most important roles of the mitochondria is the oxidation of cytosolic NADH. Since the mitochondrial inner membrane is impermeable to this molecule, the electrons are incorporated into diverse carbon intermediates, which are oxidized by the respiratory chain. The mechanisms required to accomplish this process are known as redox

shuttles, and a large diversity of them has been reported. *U. maydis* is a non-fermentative organism and it is possible that the mitochondrial oxidation of NADH represents a major pathway, so, the study the redox shuttle mechanisms found in this fungus could be interesting.

Although *U. maydis* has the genetic complement for all the reported redox shuttle mechanisms, the ND2e seems to be the major pathway, because its activity is larger compared with the other shuttles. However, the ND2e can be accompanied by the glycerol 3-phosphate shuttle, or by the acetaldehyde-ethanol or the malate-oxaloacetate shuttles, but their activities depend on particular growth conditions.

1. INTRODUCCIÓN

"Me encontraba en una lucha a muerte con el ego: Dios y Satán lucharon por mi alma durante aquellas tres largas horas. Dios ganó, y ahora sólo tengo una duda, ¿cuál de los dos era Dios?".

Aleister Crowley, 1904

1.1. La biología de *Ustilago maydis*

Ustilago maydis es un hongo fitopatógeno que infecta especies silvestres y comerciales de maíz (*Zea mays* y *Zea mexicana*), produciendo la enfermedad conocida como carbón o tizón del maíz, debido a la formación de agallas llenas de teliosporas altamente melanizadas en los órganos reproductores de la planta, con un color negro característico (Figura 1).
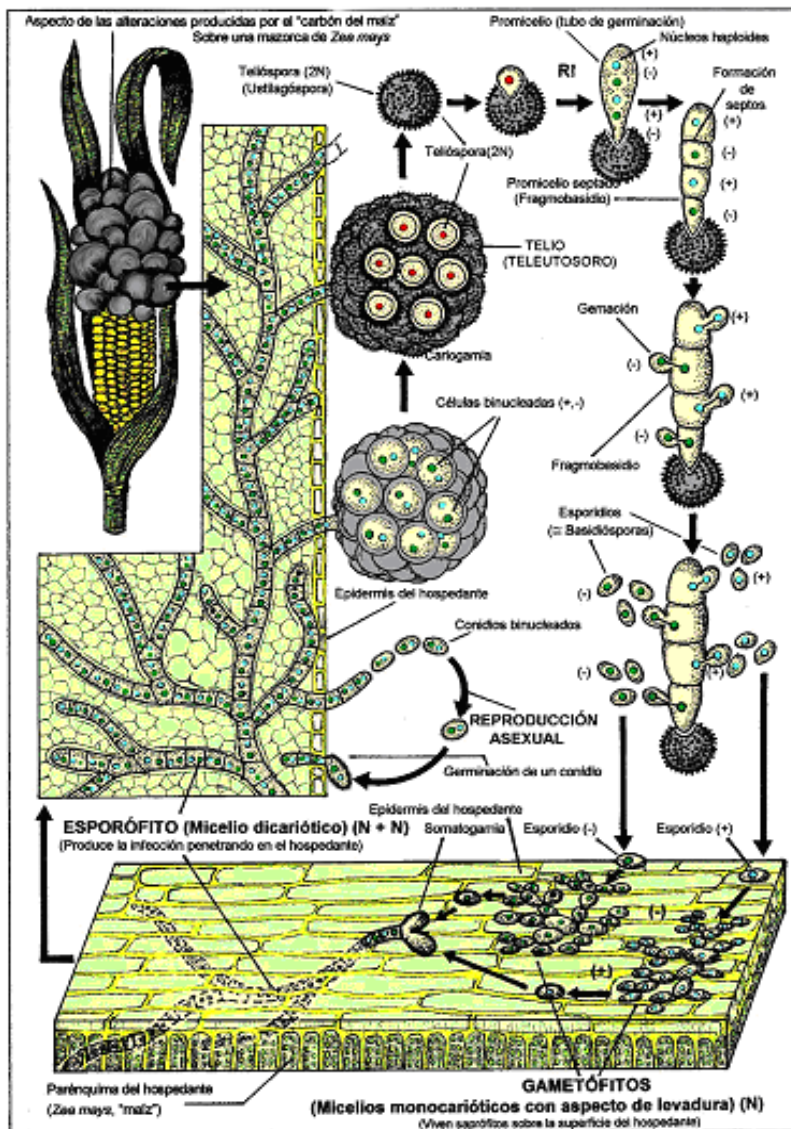
Figura 1. Ciclo de vida de *Ustilago maydis*. Tomado de la página electrónica del departamento de biología de organismos y sistemas, Universidad de Oviedo, España [http://www.uniovi.es/BOS/Asignaturas/Botanica/9.htm].

Además, infecta todas las partes aéreas de la planta, produciendo una disminución en el crecimiento del hospedero, clorosis, antocianosis y de manera interesante, produce poblaciones mixtas de flores femeninas y masculinas en los órganos donde normalmente se encuentran las flores de un sólo sexo [1, 2].

1.1.1 Clasificación taxonómica

Los hongos se clasifican en cinco grandes grupos dependiendo de sus estructuras de reproducción sexual [3]:

- Chytridiomycota. Producen zoosporas que pueden moverse independientemente, por medio de un flagelo.
- Zygomycota. Se reproducen sexualmente a través de las zigosporas, que no presentan movilidad.
- Glomeromycota. Forman las asociaciones simbiónticas llamadas micorrizas arbusculares. La mayoría de las especies se reproducen asexualmente.
- Ascomycota. Se reproducen sexualmente mediante ascosporas, las cuales se encuentran en una estructura en forma de saco, conocida como asca.
- Basidiomycota. Se reproducen sexualmente a través de las basidiosporas, que son producidas por una estructura en forma de pedestal denominada basidio (Figura 2).
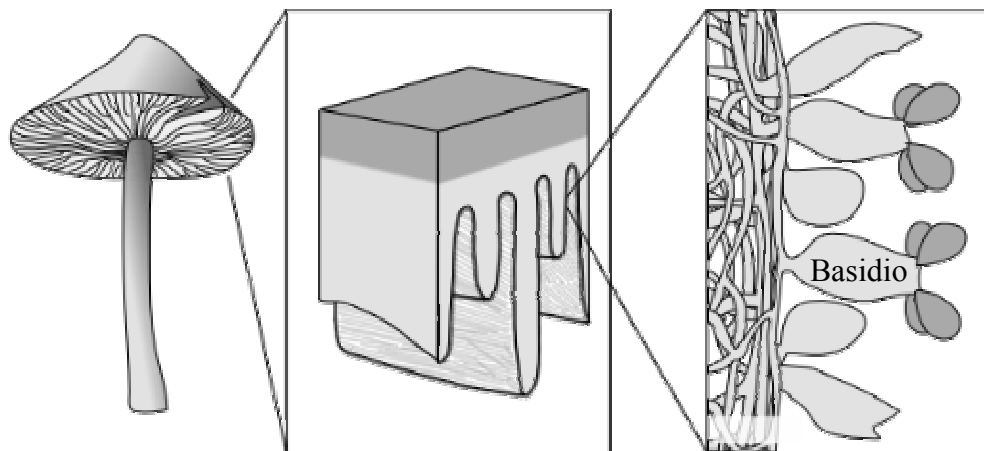
Figura 2. El basidio es una estructura microscópica que se encuentra en los extremos de las hifas situadas en los cuerpos fructíferos de los hongos basidiomicetos. En el basidio se producen las esporas sexuales llamadas basidiosporas.

La división Basidiomycota es un grupo numeroso donde se encuentran situadas más de 22,000 especies (37% de los hongos descritos). En el pasado, se dividían en Homobasidiomycetos (las setas) y Heterobasidiomycetos (las royas y los tizones). Sin embargo, en el esquema moderno se reconocen tres grupos, los Himenomicetos (setas), los Ustilaginomicetos (tizones) y los Teliomicetos (también llamados urediniomicetos o royas) [4, 5].

La clase de los Ustilaginomicetos comprende cerca de 1400 especies de hongos parásitos, que infectan a más de 70 géneros de plantas vasculares. Por ejemplo, dos especies infectan a licofitas, otra infecta helechos, dos infectan coníferas y todas las demás son parásitas de angiospermas (810 especies en poáceas y 170 en ciperáceas), lo que los hace el grupo de patógenos con mayor rango de hospedero descrito. Una característica interesante de esta clase de hongos es que las especies formadoras de teliosporas infectan herbáceas, mientras que las que no forman teliosporas suelen infectar árboles o arbustos. A pesar de que todas las especies de ustilaginomicetos esporulan sobre o dentro de los tejidos parenquimatosos, la ubicación de los soros pueden variar, encontrándose en las raíces, tallos, hojas, inflorescencias, flores, anteras, ovarios o semillas [2].

Los ustilaginomicetos se encuentran divididos a su vez en los Entorrhizomicetidae, los Ustilaginomicetidae y los Exobasidiomicetidae [6, 7]. De los tres grupos los más peligrosos son los Ustilaginomicetidae, debido a que en este clado se ubican los géneros Tilletia y Ustilago, que presentan una gran cantidad de patógenos de especies comerciales [8].

1.1.2 Ciclo de vida

El ciclo de vida de *Ustilago maydis* (Figura 1) se considera diplohaplofásico, ya que presenta una alternancia de dos generaciones morfológicamente distintas: la unicelular, denominada basidiospora (o esporidio), que es saprobia, haploide y uninucleada, y la miceliar, que es parásita y dicarionte. La forma miceliar (esporofito) predomina en tamaño y duración, por lo cual es un hongo digenético heteromórfico con esporofito dominante [1, 2, 8].

El ciclo comienza cuando las basidiosporas entran en contacto con los tejidos de la planta hospedera, usualmente a través de la dispersión por el viento o por el rebote del agua de

lluvia. En la planta, las basidiosporas forman un micelio de vida corta, que es poco infectivo (gametofito), que al ponerse en contacto con otro micelio de una cepa compatible (heterózigo en los grupos de genes A y B) produce un tubo de conjugación, por el que se lleva a cabo la migración del núcleo y posteriormente la plasmogamia, dando lugar a la formación de un micelio verdadero dicarionte, que es la forma de vida parásita de la planta y que es capaz de diseminarse hacia otros tejidos [1, 2].

El micelio dicarionte promueve la formación de agallas en los órganos reproductores femeninos del maíz, a través de la secreción de ciertos factores hormonales (principalmente auxina) [1], que producen la hipertrofia de la cubierta de los granos del maíz, dentro de los cuales el micelio se expande, y en etapas posteriores, las células dicariontes se separan del micelio, adquieren una forma redonda u ovalada, engrosan su pared y finalmente se melanizan, formando así las telisporas [1, 2, 8].

Una vez que la planta muere, la pared de la agalla se deseca y desquebraja, liberando las teliosporas, las cuales presentan varios estadios de maduración. En su etapa inmadura presentan dos núcleos y en etapas posteriores ocurre la cariogamia, por lo que las teliosporas maduras presentan un núcleo diploide. Durante la germinación se lleva a cabo la meiosis, la duplicación de los núcleos resultantes y la formación de un promicelio (o tubo de maduración), en donde los 4 núcleos se separan a través de septos, produciéndose una estructura cilíndrica ramificada con 4 células (basidio). Una vez que el basidio se ha formado, las células (gametos) sufren procesos de gemación, produciéndose las basidiosporas [2].

1.1.3 El control molecular del ciclo de vida

La transición dimórfica de *U. maydis* se lleva a cabo en dos fases, el reconocimiento y fusión de las cepas compatibles, y el mantenimiento y expansión del micelio dicarionte [9, 10]. El reconocimiento celular y la fusión están mediadas a través del locus dialélico A, el cual produce una hormona lipopeptídica (Mfa1/2) y su receptor (Pra1/2) [9-12]. Como resultado de la interacción del receptor con la feromona, las células son detenidas en la etapa G_0 del ciclo celular y se lleva a cabo la formación del tubo de conjugación, que se orienta hacia la fuente del estímulo hormonal [10]. De manera paralela, se promueve la

transcripción de varios genes, en particular de una secuencia pequeña de RNA denominada "elemento de respuesta a las feromonas", que se une en los extremos 5´ o 3´ y regula la expresión de los genes inducibles por feromonas. Dentro de este tipo de genes se encuentra el factor transcripcional prf1, que coordina la respuesta a las feromonas y la expresión de los genes del locus B [9].

Cuando los tubos de conjugación entran en contacto se lleva a cabo la fusión celular y la migración de los núcleos. En esta etapa se realiza la verificación de la heterozigosis en el locus B, lo que conlleva a la formación y expansión del micelio. El locus B muestra más de 25 alelos y codifica para dos proteínas de la familia de los homeodominios, bE y bW, que actúan como regulador transcripcional [9-12], activando la expresión de los genes de patogenicidad, y en última instancia controlan el cambio dimófico [10, 11]. El mecanismo que asegura la heterozigosis en este locus es la formación de heterodímeros de las proteínas bE y bW, lo cual ocurre únicamente cuando los alelos de los dos genes son distintos. El reconocimiento isomórfico está determinado por una sección de 100 aminoácidos en el extremo amino terminal [13].
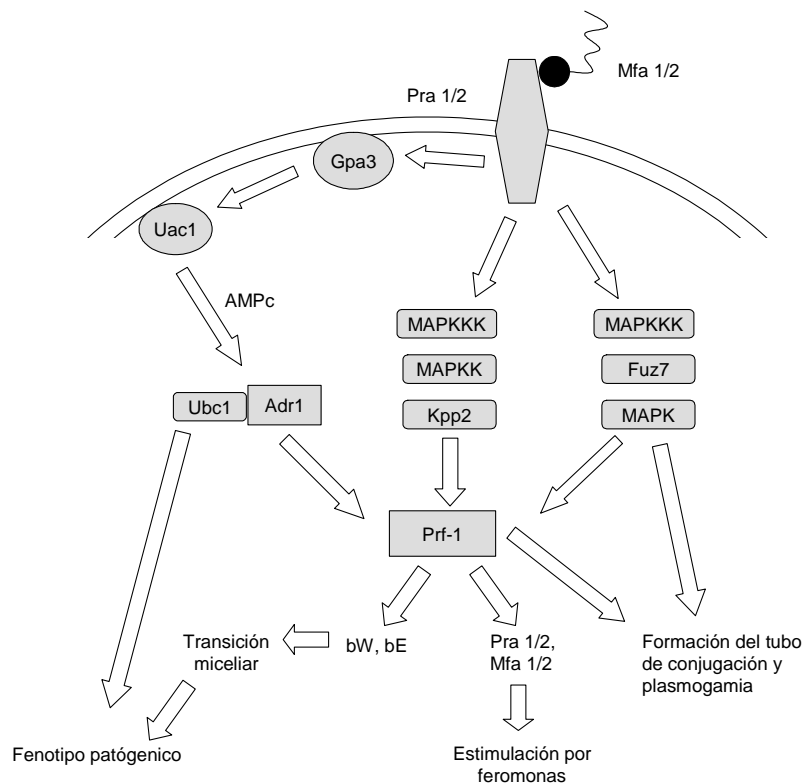
Figura 3. Rutas de señalización utilizadas durante el apareamiento celular y la transición dimórfica de *Ustilago maydis* [9].

De manera adicional al control transcripcional, se reconoce que la señalización intracelular es uno de los factores más importantes que controlan y coordinan diversos procesos en la conjugación (figura 3). En particular, se ha descrito que la interacción del receptor con su ligando activa tres vías de señalización, compuestas por dos módulos de MAP cinasas, que tienen como intermediario, ya sea a Kpp2 o a Fuz7 [9, 11], y una vía dependiente de AMPc, que se activa a través de una proteína G acoplada al receptor (Gpa3), que a su vez estimula a la adenilato ciclasa membranal (Uac1), la cual incrementa la concentración de AMPc, promoviendo la disociacón de la PKA (Adr-1) y su subunidad reguladora (Ubc-1), lo que estimula la fosforilación de una gran variedad de blancos. La activación de las tres vías conlleva a la fosforilación de Prf-1, que activa una serie de procesos, como la transcripción, la conjugación, la formación del tubo, la fusión celular o la patogénesis [9-11].

1.1.4 Importancia económica

U. maydis produce pérdidas significativas en la producción de diversas variedades de maíz en un gran número de países [8], debido en gran medida al sistema de monocultivo operante y a que las variedades más rentables no presentan resistencia natural a la infección. A pesar de ésto, *U. maydis* no es el basidiomiceto más peligroso desde el punto de vista económico, ya que otros hongos Ustilaginales del género Ustilago o Tilletia llegan a producir pérdidas de hasta el 100% en diversos cultivares, como el sorgo, la avena, la cebada, la caña de azúcar y algunas variedades de trigo, entre otros [2].

Por otro lado, en la meseta central y en el sur de México se reconoce al carbón del maíz como una valiosa fuente nutricional en la dieta de las poblaciones que no tienen acceso ontinuo a las proteínas de origen animal [2], y actualmente otros países, principalmente asiáticos, reconocen el valor alimenticio de *U. maydis*, así como su sabor característico. Sin embargo, su aceptación en el mercado mundial está limitada por su apariencia y por su reputación como plaga.

1.1.5 *Ustilago maydis* como modelo de estudio.

Este organismo tiene una gran cantidad de ventajas por las cuales ha sido usado para estudiar una diversidad de procesos biológicos, tales como la recombinación genética, la reparación del DNA, el apareamiento de gametos, la morfogénesis, la resistencia a fungicidas y los procesos de desarrollo de la patogenicidad, por lo cual es considerado un organismo fitopatógeno modelo [1, 2, 8]. Dentro de las ventajas que ofrece se encuentran: a) que a diferencia de la mayoría los hongos patógenos, tanto su fase saprobia como parasita pueden ser cultivadas *in vitro*; b) que la fase haploide puede cultivarse en medio sólido en colonias compactas, lo cual permite el uso de técnicas microbiológicas estándar; c) que la transición dimórfica se encuentra regulada por un control maestro único y no por una multiplicidad de genes, como ocurre en otros patógenos, por lo cual el análisis de los procesos de patogenicidad y la identificación de los genes involucrados se facilita; d) que la producción de cepas knockout es relativamente sencilla, debido a la alta proporción con la que la recombinación homóloga se lleva a cabo en este hongo; e) que se pueden producir cepas estables de células knockout para genes esenciales, debido a la complementación genética de la fase diploide; f) que la presencia de varios factores genéticos dominantes permite la selección de colonias con modificaciones genéticas, y finalmente g) que se pueden realizar análisis de segregación alélica, ya que este organismo alterna entre las fases haploide y diploide, por lo que la meiosis y el apareamiento de los gametos son procesos que ocurren frecuentemente y que pueden ser controlados *in vitro*.

1.2 La fosforilación oxidativa

La evidencia geológica indica que la atmósfera estaba compuesta principalmente por gases como el N_2 y el H_2, en las etapas en las que se originó la vida, y abundaban otras especies químicas, como el metano y el amonio, lo que volvía al medio primitivo un ambiente reductor [19, 20]. Aproximadamente hace 3500 millones de años aparecieron las primeras cianobacterias [21], las cuales desarrollaron el proceso de la fotosíntesis, una vía metabólica que oxida al agua, produciendo equivalentes reductores (utilizados con fines biosintéticos) y oxígeno molecular. Estos organismos provocaron que los niveles atmosféricos de O_2 se elevaran enormemente en un intervalo corto, lo que condujo a que el resto de los organismos desarrollaran sistemas de protección contra este nuevo gas [22]. A pesar de que el O_2 tiene una reactividad relativamente baja, resulta tóxico debido sus características químicas, como son: a) es un birradical, que puede ser convertido con facilidad en otras especies mucho más reactivas, como el oxígeno en singulete, y b) que sus dos electrones no apareados presentan espines paralelos, por lo que en la eliminación de este gas se produce una situación paradójica, ya que la transferencia de electrones es forzosamente univalente, lo cual genera otras especies radicales aún más tóxicas que el propio O_2 [23].

Dentro de las estrategias que se produjeron para sobrevivir en el ambiente oxidativo, se encuentran la eliminación de la especies reactivas y la reparación del daño que producen. Sin embargo, muchos organismos se volvieron capaces de utilizar el poder oxidante de este gas para sintetizar ATP, mediante el aparato metabólico conocido como fosforilación oxidativa, la cual está compuesta por dos módulos, el sistema oxidativo y el sistema fosforilante [24]. El sistema oxidativo a su vez está compuesto por los cuatro complejos enzimáticos de la cadena respiratoria y por otras enzimas del tipo de las deshidrogenasas que ceden los electrones a nivel de la poza de ubiquinona, como la glicerol 3-fosfato deshidrogenasa mitocondrial o la acil-CoA deshidrogenasa. El sistema fosforilante está compuesto por el acarreador de fosfato, la adenín nucleótido translocasa y el complejo V, también conocido como ATP sintetasa.

1.2.1 Aspectos termodinámicos de la síntesis de ATP

En un ambiente con un alto contenido de oxígeno las reacciones de oxidación se encuentran favorecidas termodinámicamente, por lo que durante la transferencia de electrones hacia el aceptor final se libera energía libre, que puede ser utilizada para realizar trabajo, como el transporte de metabolitos o proteínas, o la síntesis de ATP. Por ejemplo, durante la oxidación completa del NADH se producen 218 kJ/ mol, con lo que se podrían sintetizar poco más de siete moles de ATP, y que corresponde a la energía necesaria para levantar 21.8 toneladas a un metro del suelo.

Con la propuesta de la teoría quimiosmótica de Mitchell [25] se aclaró el mecanismo por el cual el módulo oxidativo y el módulo fosforilante se coordinan para realizar la síntesis de ATP. La energía libre, que se obtiene en los procesos de oxidación, es utilizada por la cadena respiratoria para bombear protones a través de la membrana interna mitocondrial (o la membrana plasmática en las bacterias), generando así una diferencia de potencial electroquímico, la cual es una forma de almacenar una parte de la energía liberada por la transferencia de electrones (Figura 3).
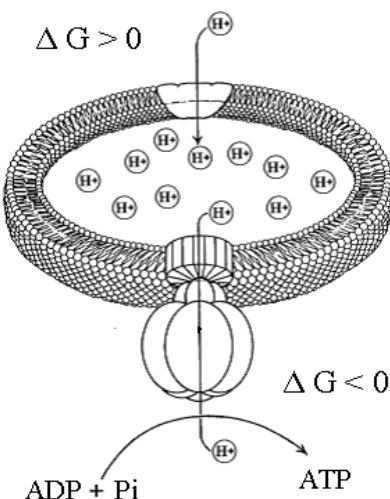
Figura 3. Mecanismo general de la síntesis de ATP por la fosforilación oxidativa. El valor relativo del $\Delta G°$ corresponde exclusivamente al transporte de protones.

Por su parte, la ATP sintetasa funciona a manera de compuerta o canal, dejando pasar los protones (disipando el potencial) y utilizando la energía que se libera en este proceso de transporte para sintetizar el ATP.

1.2.1. El sistema oxidativo

La oxidación de compuestos como el NADH, el succinato o el glicerol 3-fosfato se lleva a cabo a través de las enzimas de la cadena respiratoria, que presentan diversos grupos prostéticos con valores decrecientes escalonados de potencial redox (Tabla I), a través de los cuales los electrones fluyen hasta el aceptor final, que es el O_2.

Complejo	Grupo prostético	$E°'$ (mV)
NADH		-315
Complejo I (NADH: ubiquinona oxidoreductasa)		
	FMN	¿?
	N-1ª	-380
	N-1b	-250
	N-2	-30
	N-3	-245
	N-4	-250
	N-5	-270
	N-6	-275
Succinato		30
Complejo II (Succinato: ubiquinona oxidoreductasa)		
	FAD	-40
	S-1	-30
	S-2	-245
	S-3	60
	Citocromo b_{560}	-80
Ubiquinona		45
Complejo III (Ubiquinol: citocromo c oxidoreductasa)		
	Citocromo b_H	30
	Citocromo b_L	-30
	Fe-S	280
	Citocromo c_1	215
Citocromo c		235
Complejo IV (citocromo c oxidasa)		
	Citocromo a	210
	Cu_A	245
	Cu_B	340
	Citocromo a_3	385
O_2		815

Tabla I. Potencial redox estandar ($E°'$) de los grupos prostéticos e intermediarios de la cadena transportadora de electrones [26].

Como se mencionó, la sección central del sistema oxidativo está compuesta por los cuatro complejos de la cadena transportadora de electrones (Figura 4).
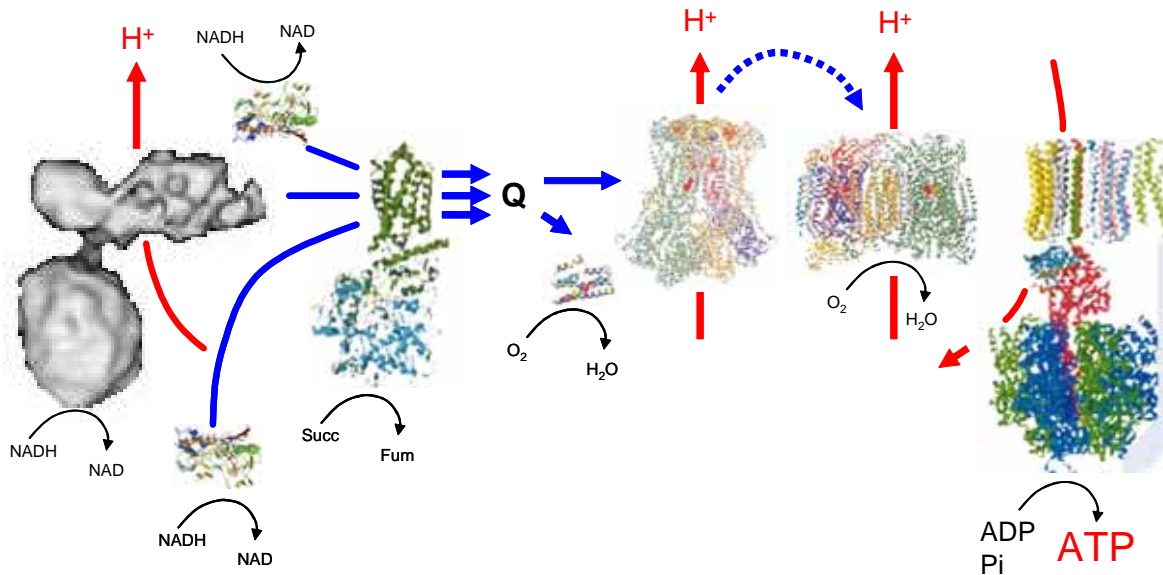
Figura 4. Cadena transportadora de electrones de tipo eucarionte, donde se muestran las rutas clásicas y alternas de reducción y oxidación de la poza de ubiquinona.

1.2.1.1. El complejo I

Esta enzima cataliza la transferencia de electrones del NADH a la molécula de ubiquinona (Q), de acuerdo con la siguiente estequiometría:

$$NADH + Q + 5H^+_{in} \rightarrow NAD^+ + QH_2 + 4H^+_{ex} \qquad \underline{\Delta G\ ^{\circ\prime} = -69.5\ kJ/\ mol}$$

En esta reacción cuatro protones son bombeados a través de la bicapa lipídica membranal, debido a la gran cantidad de energía libre desprendida durante el proceso redox (nota: los valores de $\Delta G\ ^{\circ\prime}$ que se muestran no contemplan el bombeo de protones, debido a que la energía necesaria para mover iones depende del potencial transmembranal).

En los mamíferos, el complejo I está compuesto de al menos 43 subunidades, de las cuales siete son codificadas en el interior de la mitocondria y el resto por el DNA nuclear [27]. En

las bacterias, esta enzima está compuesta de 13-14 subunidades, por lo que se considera a este arreglo la unidad funcional mínima [28] (Figura 5).
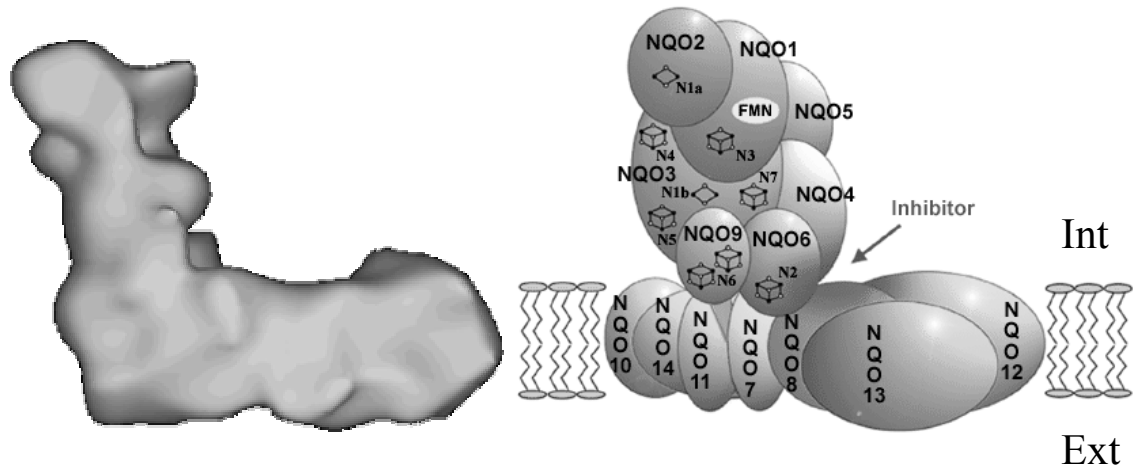
Figura 5. Estructura tridimensional del complejo I. A la Izquierda se muestra una reconstrucción de imagen de fotografías obtenidas mediante microscopía electrónica, del complejo I de *Neurospora crassa*. A la derecha se muestra un esquema hipotético donde se indica la posición de las subunidades del complejo I, así como de los grupos prostéticos. Ext, exterior (o lado positivo de la membrana); Int, interior (o lado negativo de la membrana) [29].

El complejo I está formado por el dominio membranal, donde se encuentran localizadas la subunidades hidrofóbicas (incluyendo las que se sintetizan en la mitocondria), y el dominio periférico, reconocido como la sección catalítica de la proteína [24]. Presenta varios cofactores, dentro de los que están una molécula de FMN unida de manera no covalente y de 8 a 10 centros Fe-S, de los cuales los centros N1a y N1b son binucleares, mientras que los centros N2, N3, N4, N5 y N6 son tetranucleares [30]. Además, en algunas bacterias se han reportado los centros tetranucleares N6b y N7, y de hecho se ha podido discernir su ubicación en el segmento periférico del complejo I de *Escherichia coli* (Figura 6) [31].

Por otro lado, se han propuesto dos mecanismos generales para explicar la translocación de protones en la cadena respiratoria: el asa redox, propuesta por Mitchell [32], y la bomba de protones, propuesta por Chance [33]. En el mecanismo del asa redox, los eventos de transferencia de electrones son acompañados por la protonación y desprotonación de los

grupos prostéticos (como el FMN) o sustratos (como la ubiquinona), con la singularidad de que la protonación ocurre exclusivamente del lado interno, y la desprotonación en el lado externo, por lo que el transporte de electrones conlleva a la producción del gradiente iónico. En la hipótesis de la bomba de protones, se propone que los complejos respiratorios presentan un canal perpendicular al plano de la membrana, formado por residuos polares, los cuales sufren modificaciones en sus pKa durante la transferencia interna de electrones, lo que promueve que los protones se muevan a través del canal.
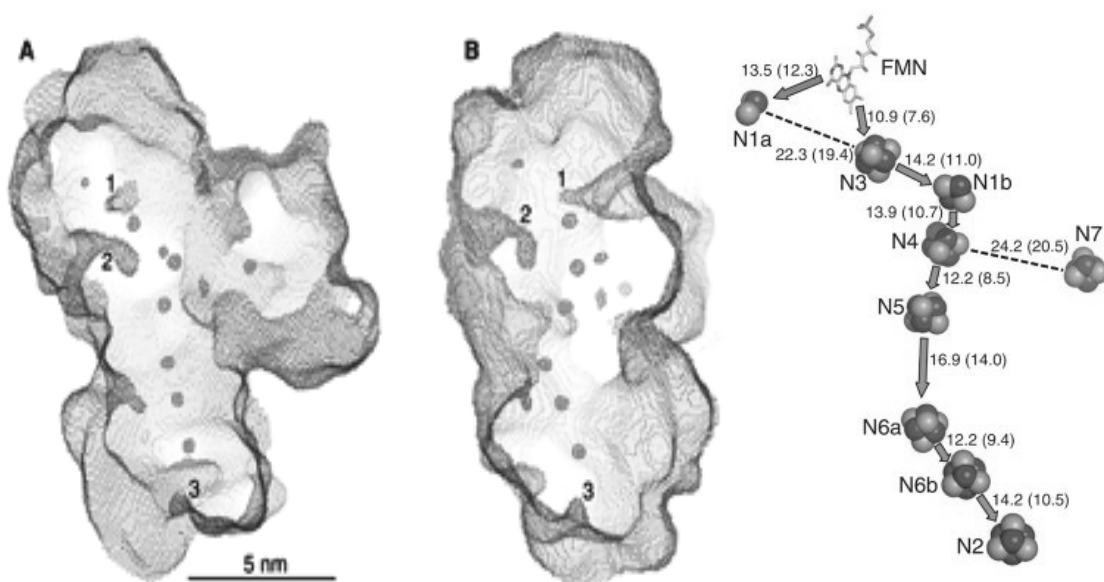
Figura 6. Localización de los centros Fe-S en el dominio periférico del complejo I de la bacteria gram negativa *E. coli*.

Se desconoce el mecanismo de bombeo de protones en el complejo I, en gran medida debido a que su estructura tridimensional no está resuelta, en particular los sitios de unión de la ubiquinona. Sin embargo, se han sugerido modificaciones al modelo del asa redox para explicar el proceso de translocación de protones, en donde se considera que ya sea la ubiquinona (Figura 7) [34-37], el FMN [37] y/ o el centro N2 [36, 37] son los grupos protonables, aunque no se tiene certeza sobre cuales de ellos participan.
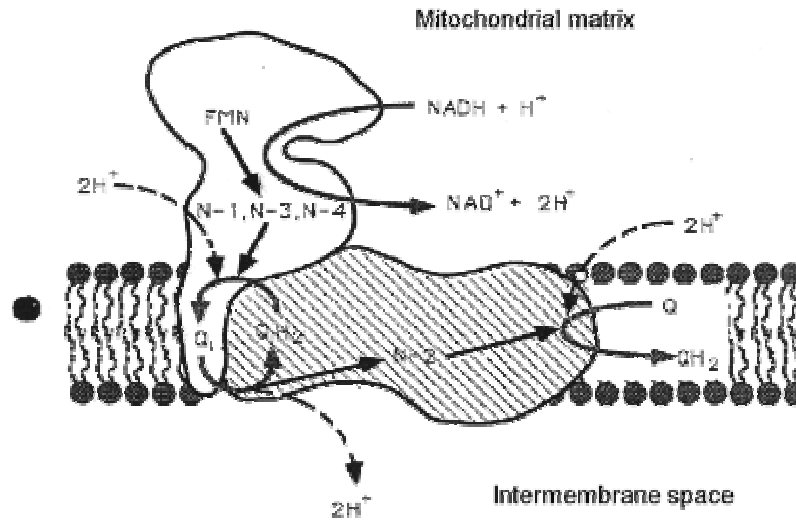
Figura 7. Mecanismo modificado de tipo asa redox propuesto para explicar el bombeo de protones en el complejo I, en donde el acarredor de electrones y protones es la molécula de ubiquinona.

1.2.1.2. El complejo II

Este complejo cataliza la transferencia de electrones del succinato a la ubiquinona y le brinda a la mitocondria un vínculo directo entre el ciclo de Krebs y la cadena respiratoria:

$$\text{Succinato} + Q \rightarrow \text{Fumarato} + QH_2 \qquad\qquad \Delta G\,°´ = \text{-2.9 kJ/ mol}$$

Debido a que el potencial redox del par succinato/ fumarato es muy parecido al de ubiquinona/ ubiquinol, no se libera la energía suficiente para bombear protones [24], por lo que esta enzima no participa directamente en la síntesis de ATP.

La succinato deshidrogenasa está compuesta de 4 subunidades denominadas SdhA, SdhB, SdhC y SdhD, que conforman el dominio soluble e hidrofóbico (Figura 8). El dominio soluble está compuesto por las subunidades SdhA y SdhB, es catalíticamente activo y presenta el sitio de unión del succinato, la molécula de FAD y los tres centros Fe-S. El dominio hidrofóbico está compuesto por las subunidades C y D, presenta al hemo b_{560} y el sitio de unión de la ubiquinona [24, 38].
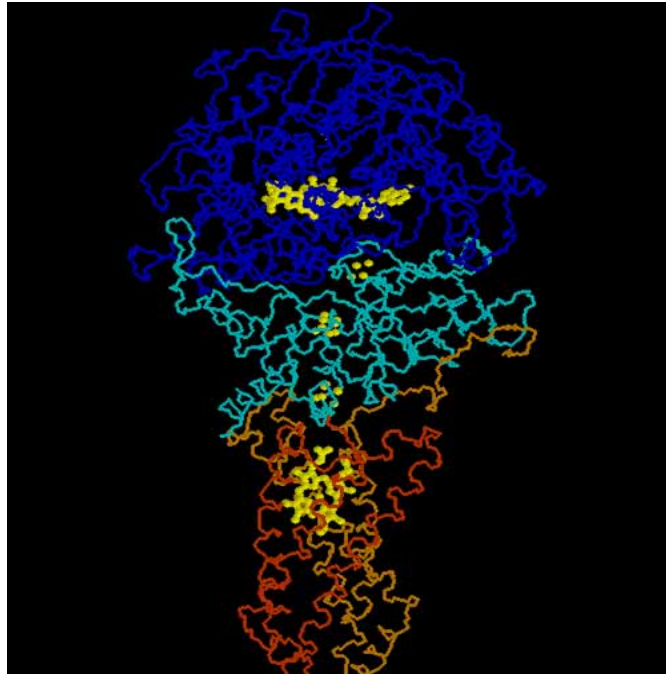
Figura 8. Estructura tridimensional del complejo II. En azul marino se muestra la subunidad A, conteniendo la molécula de FAD. En azul claro se subunidad B con los centros Fe-S. En rojo y anaranjado las subunidades C y D con el hemo b_{560}. Los grupos protéticos son mostrados en color amarillo.

Durante la reacción oxidativa, los dos electrones del succinato son transferidos de manera simultánea a la molécula de FAD, para posteriormente reducir a los centros Fe-S y al hemo b_{560}, el cual finalmente los dona a la molécula de ubiquinona [24]. Es importante hacer notar que el centro S-2 tiene un potencial redox muy negativo (-245 mV) [39], lo que sugiere que no tiene participación en el transporte de electrones. De hecho, este centro se detecta exclusivamente en la enzima purificada, pero no en las preparaciones mitocondriales crudas [40].

1.2.1.3. El complejo III

Esta enzima transfiere los electrones de la molécula de ubiquinol (QH_2) hacia el citocromo c, bombeando 4 protones:

QH_2 + 2 citocromo c_{ox} + 2H^+_{in} → Q + 2 citocromo c_{red} + 4 H^+_{ex} $\quad$ $\underline{\Delta G\,^{\circ}{'} = -36.7\ kJ/\ mol}$

El complejo III en los mamíferos está formado por 10 subunidades, de las cuales nueve son sintetizadas en el citosol y una (citocromo b) en la matriz mitocondrial [24]. Las subunidades catalíticas son el citocromo b, la proteína Fe-S y el citocromo c_1 (Figura 9).
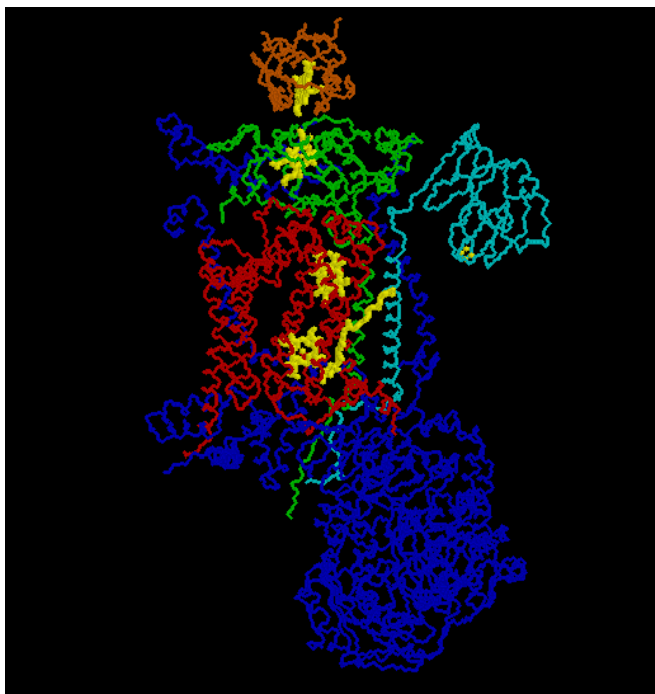
Figura 9. Estructura tridimensional del complejo III. En azul marino se muestran las subunidades supernumerarias. En rojo se muestra al citocromo b con los hemos b_L (arriba) y b_H (abajo). En azul claro se muestra la proteína Rieske con el centro Fe-S. En verde se aprecia el citocromo c_1 y en anaranjado el citocromo c. Los grupos protéticos son mostrados en color amarillo.

El citocromo b es una proteína integral de membrana que presenta dos hemos de tipo b que difieren en su posición y en su potencial redox [41, 42]. El hemo b_L tiene un potencial de -30 mV y se encuentra orientado hacia el espacio intermembranal (exterior), mientras que el hemo b_H tiene un potencial de 30 mV y se encuentra orientado hacia la matriz mitocondrial. En el citocromo b también se encuentran los sitios de unión de dos moléculas de ubiquinona, conocidos como los sitios Qo y Qi. El sitio Qo que se encuentra adyacente al hemo b_L y el Qi en cercania con el hemo b_H.

La proteína Fe-S presenta un segmento hidrofóbico, que atraviesa la membrana interna mitocondrial y que forma parte importante de las interacciones que permiten que el

complejo III se dimerice [43]. También presenta un dominio soluble, donde se encuentra el centro binuclear Fe-S, que durante el ciclo catalítico alterna su posición entre el sitio Qo y el citocromo c_1. Por su parte, el citocromo c_1, al igual que la proteína Fe-S, tiene un segmento transmembranal y uno soluble, en donde se encuentra el hemo c_1, en una sección próxima al sitio de unión del citocromo c.
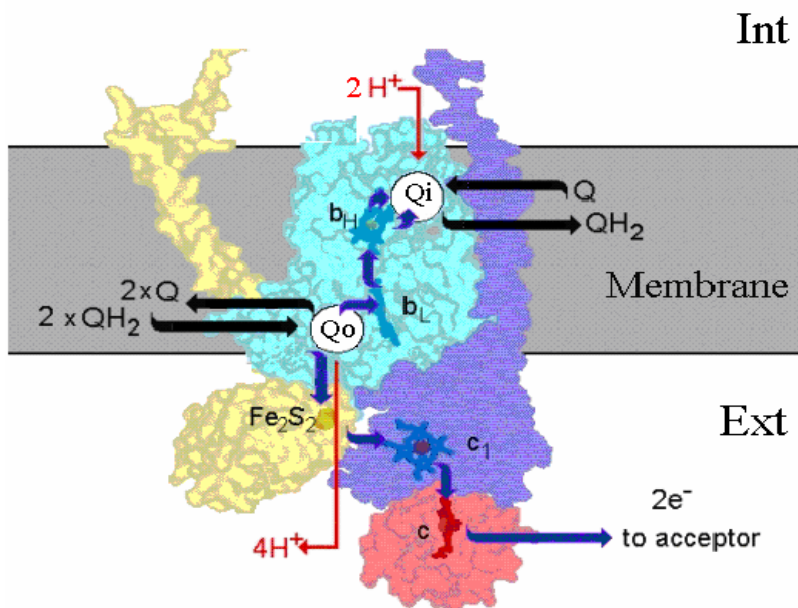
Figura 10. Mecanismo de reacción del complejo III, mostrando las reacciones del ciclo Q.

El complejo III lleva a cabo el bombeo de protones a través de un mecanismo semejante al asa redox de Mitchell, conocido como el ciclo Q (Figura 10) [44, 45]. Para que el ciclo catalítico de la enzima se complete se requiere que el ciclo Q se lleve a cabo dos veces. En la primera vuelta, el ubiquinol es oxidado y desprotonado en el sitio Qo, y los electrones son separados por dos vías diferentes, en una de ellas el electrón es transferido a la proteína Fe-S, para posteriormente ser entregado al citocromo c_1 y luego al citocromo c. En la otra vía el electrón es transferido del hemo b_L, posteriormente al hemo b_H y finalmente a una molécula de ubiquinona, unida al sitio Qi, formándose un radical semiquinona. En la segunda vuelta del ciclo, otra molécula de ubiquinol es oxidada y nuevamente uno de los dos electrones pasa al citocromo c, y el otro se transfiere a la semiquinona, la cual se protona del lado de la matriz mitocondrial y se libera en forma de ubiquinol. Una vez que se completaron las dos vueltas del ciclo Q, se transfirieron dos electrones a la poza de

24

citocromo c, se translocaron cuantro protones al espacio intermembranal y se consumieron dos del lado de la matriz.

1.2.1.4. El complejo IV

Esta enzima cataliza la transferencia de electrones del citocromo c reducido hacia el oxígeno molecular, de acuerdo con la siguiente reacción:

$$4 \text{ citocromo } c_{red} + 8H^+_{in} + O_2 \rightarrow 4 \text{ citocromo } c_{ox} + 2H_2O + 4H^+_{ex} \quad \underline{\Delta G\,°´ = -112 \text{ kJ/ mol}}$$
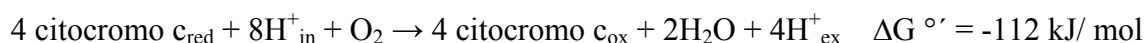
En los mamíferos, el complejo IV está compuesto de diez subunidades de origen nuclear y tres sintetizadas intramitocondrialmente (COXI, COXII y COXIII) [24]. Sin embargo, sólo dos de las subunidades catalizan procesos de transferencia de eletrones. La subunidad I contiene el centro de Cu_B y los hemos a y a_3 y la subunidad II presenta el sitio de unión del citocromo c y el centro binuclear Cu_A (Figura 11) [24, 46, 47].

La transferencia de electrones se inicia cuando el Cu_A toma el electrón del citocromo c reducido y se lo transfiere al hemo a, para que posteriormente éste lo transfiera al centro binuclear compuesto por el hemo a_3 y el Cu_B, donde se llevan a cabo cuatro reducciones univalentes del oxígeno molecular y finalmente se liberan dos moléculas de agua [46, 47].

Uno de los esquemas con mayor aceptación, que explica el mecanismo de bombeo de protones por el complejo IV, sugiere que la enzima en su estado oxidado se protona en un residuo de ácido aspártico que forma parte del canal D, el cual desemboca en el centro binuclear formado por el Cu_B y el hemo a_3 (Figura 12). Durante la transferencia interna de electrones, los residuos del canal cambian el valor de sus pKa, lo que permite que el protón sea transferido a un residuo de histidina que coordina al Cu_B. La evidencia cristalográfica indica que esta histidina presenta una gran movilidad y se propone que puede cambiar su posición y orientarse hacia otro canal, formado por residuos acídicos con valores de pKa bajos, a través del cual, el protón es liberado al espacio externo.

Cabe resaltar que el complejo IV también presenta un canal denominado K, pero actualmente se reconoce que por éste pasan los H^+ que se consumen en la formación de las moléculas de agua [48, 49].
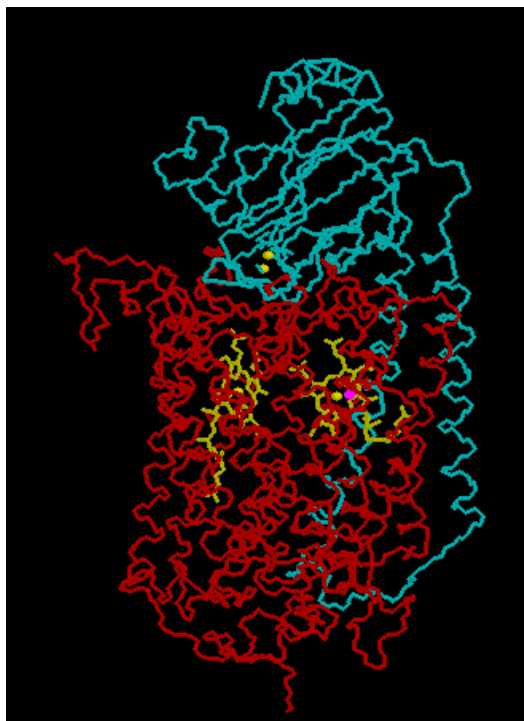
Figura 11. Estructura tridimensional del complejo IV. En rojo se muestra se muestra la subunidad I, con el Cu_B y los hemos a y a_3. En azul claro se muestra la subunidad B con el centro Cu_A. El Cu_B .y los hemos a y a_3 son mostrados en color amarillo y el Cu_B es mostrado en rosa.
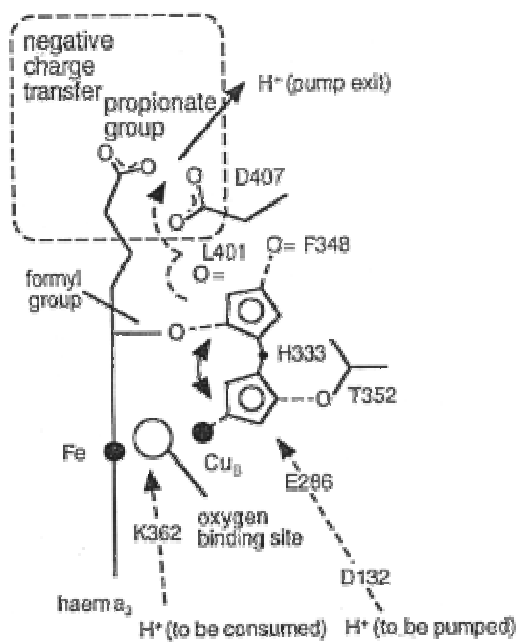
Figura 12. Mecanismo de bombeo de protones propuesto para el complejo IV.

1.2.2. El sistema fosforilante

Es a través del sistema fosforilante que la mitocondria puede sintetizar ATP. Como se mencionó, este sistema consta del complejo V, de la translocasa de adenín nucleótidos (ANT) y del acarreador de fosfato. La ANT realiza el intercambio electrogénico y reversible de una molécula de ADP por una molécula de ATP a través de la membrana interna mitocondrial. Este proceso está impulsado por la diferencia de potencial transmembranal de protones, que favorece el intercambio de una molécula con carga neta de -3 (ADP), por otra con carga neta de -4 (ATP) [24, 50]. El acarreador de fosfato realiza la incorporación de fosfato inorgánico (HPO_4^{2-}) a la mitocondria, acoplado con la entrada de un protón [24].

1.2.2.1. El complejo V

En los mamíferos el complejo V está formado por 14 subunidades, de las cuales dos (ATPase8 y ATPase6) son sintetizadas en el interior de la mitocondria [24]. Esta enzima cataliza la formación de un enlace fosfodiéster entre la molécula de ADP y el fosfato inorgánico:

$$ADP + HPO_4^{2-} + H^+ \rightarrow ATP + H_2O \qquad \Delta G\,^{\circ\prime} = 30\ kJ/\,mol$$

Como se puede observar, la reacción está desfavorecida termodinámicamente, por lo que se requiere una fuente externa de energía para que el proceso se lleve a cabo. La manera de obtener esta energía es a través de la apertura de un canal, que disipa de manera controlada el gradiente de protones. Suponiendo que el sistema oxidativo mitocondrial genera un ΔpH de -1.4 y un $\Delta \Psi$ de -140 mV, la energía liberada ($\Delta G\,^{\circ}$) por el flujo de un mol de protones hacia la matriz sería de -21.5 kJ. Como resultado, la formación del enlace fosfodiéster, acoplada al transporte de tres protones, está favorecida termodinámicamente.

$$ADP + HPO_4^{2-} + 4H^+_{ex} \leftrightarrow ATP + H_2O + 3H^+_{in} \qquad \Delta G\,^{\circ\prime} = -33.7\ kJ/\,mol$$

El complejo V presenta una estructura asimétrica compuesta por dos dominios multiprotéicos con funciones diferentes, conocidos como el dominio F_1 y el F_0 (Figura 13).
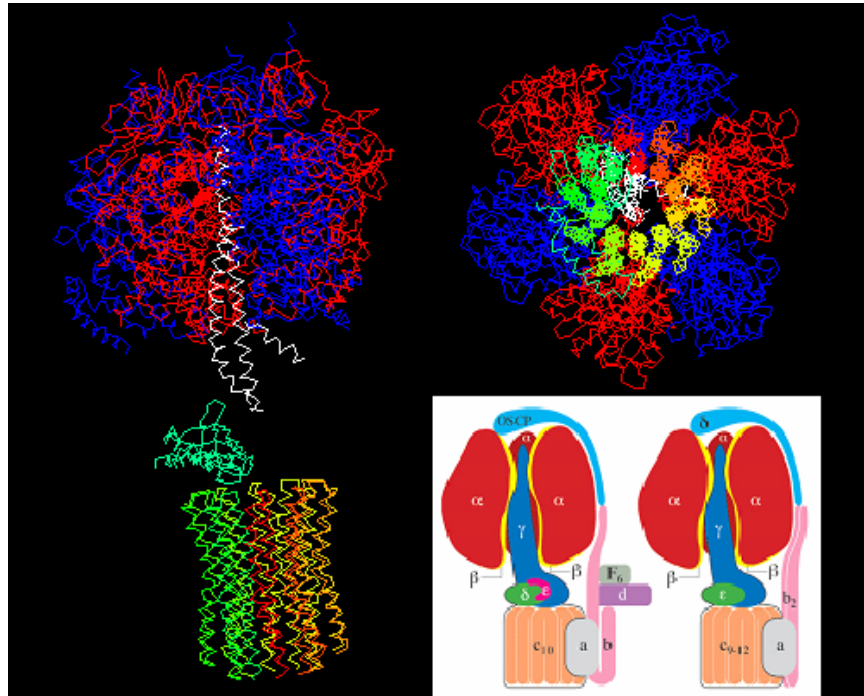
Figura 13. Estructura tridimensional del complejo V. Arriba a la derecha se muestra una vista de la parte inferior de la enzima, con el anillo de subunidades c viendo al frente. Las subunidades α se muestran en rojo, las β en azul, la subunidad γ en blanco, la ε en verde-azulado y las subunidades c se muestran en la gama de colores que van del verde al rojo. Abajo a la derecha se muestra un esquema hipotético del complejo V de bacterias y abajo a la izquierda se muestra el de los mamíferos.

El dominio F_1 es soluble, con una estructura semejante a la de una perilla y es donde se realiza la síntesis (o hidrólisis) del ATP. La estructura central del dominio F_1 la constituyen tres subunidades α y tres β, que se arreglan alternadamente en un heterohexámero, formado una estructura semejante a los gajos de una naranja. La subunidad γ atraviesa el heterohexámero $\alpha_3\beta_3$ y se une a la porción F_0, a través del anillo de subunidades c [51]. En los mamíferos se encuentran algunas subunidades accesorias como la OSCP, y las subunidades δ y ε [51].

El dominio F_0 es hidrofóbico y está compuesto de las subunidades a, b y c, mostrando una estequiometría 1: 2: 9-12. La subunidad c se pliega formando una estructura hélice –asa – hélice, que atraviesa dos veces la membrana y que se oligomeriza formando un anillo de 9 a 12 subunidades. En el interior del anillo se une la subunidad γ, con lo cual se forma el

complejo funcional F_1 -F_0 [51]. En los mamíferos también se pueden encontrar las subunidades d y F_6. Adicionalmente, se ha descrito que en el proceso de dimerización del complejo V intervienen tres subunidades supernumerarias, denominadas e, g y k, con una estequiometría de 2:2:1 por dímero. De estas tres se reconoce que las subunidades e y g son indispensables para la oligomerización, que posiblemente se lleva a cabo a través del dominio F_0 [52].

Se tiene evidencia que el complejo V tiene dos ejes verticales; el rotor, compuesto por la subunidad γ y el anillo de subunidades c, y el estator, que está formado por las subunidades a y b. La subunidad a se arregla en torno al anillo de subunidades c, y por su parte la subunidad b atraviesa la membrana, uniéndose a la subunidad a y al dominio F_1, posiblemente a traves de la subunidad β y/o δ [51].

En el proceso catalítico, la subunidad γ y el anillo de subunidades c rotan con respecto al resto del complejo, utilizando la energía libre que se obtiene de la disipación del gradiente. Una de las propuestas más aceptadas sobre el proceso de rotación indica que la subunidad a forma dos semicanales separados físicamente, uno de ellos se abre al exterior (P) de la mitocondria y el otro hacia el interior (N). Cuando un protón entra por el semicanal P, neutraliza la carga de un residuo ácido de una de las subunidades c y así ésta puede rotar, alejándose de la subunidad a y llevando a otra subunidad c sin carga hacia el semicanal N, en donde el residuo ácido se desprotona (Figura 14) [51].
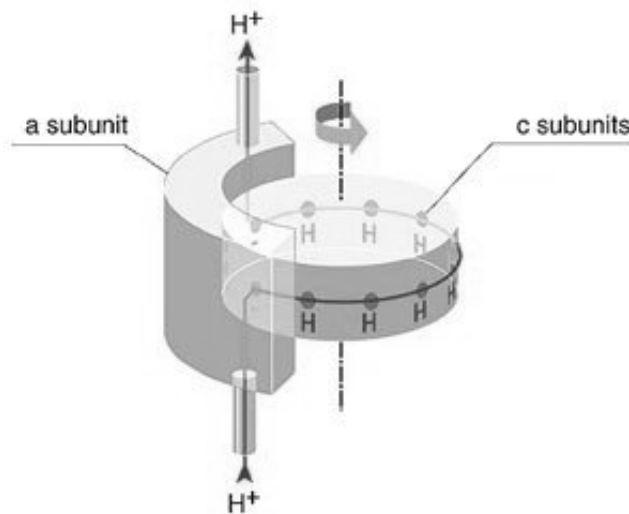
Figura 14. Mecanismo de rotación del anillo de subunidades c. El semicanal P se muestra abajo y el N arriba.

Es importante subrayar que el complejo V también puede funcionar en el sentido contrario (siempre y cuando el gradiente de protones sea bajo). De hecho, en algunas bacterias el ATP que se obtiene en la fermentación es utilizado para producir el gradiente de protones, requerido para transportar sustratos y mantener el balance iónico de la célula.

Hace más de 30 años Paul Boyer desarrolló una teoría para explicar el mecanismo de síntesis de ATP, que se denominó mecanismo de catálisis rotatoria o de cambio de afinidad [51], en el cual se propone que la síntesis de ATP se acopla a un cambio estructural en el complejo V, producido por la rotación de la subunidad γ. Cuando la subunidad γ rota, cambia su posición con respecto al heterohexámero, lo que produce que los tres sitios de unión de los sustratos, ubicados en las interfaces αβ, cambien su conformación alternadamente entre tres posibilidades: el sitio abierto, el relajado y el cerrado, de manera que en un momento dado se pueden encontrar las tres conformaciones en la enzima. El sitio relajado une con una afinidad apreciable al ADP y al fosfato, y al darse la rotación, cambia su conformación al estado cerrado, en el que se produce un ambiente hidrofóbico, donde la formación del enlace fosfodiéster se lleva a cabo de manera espontánea. Al continuar la rotación, el sitio pasa al estado abierto y el ATP es liberado [51].

1.2.3. Elementos accesorios de la fosforilación oxidativa.

Se ha descrito que una gran variedad de organismos presentan enzimas respiratorias accesorias, que ramifican la vía del transporte de electrones. Los elementos respiratorios accesorios (o alternos) pueden clasificarse en dos grupos, los que bombean iones y los que no lo hacen. Las enzimas accesorias que bombean iones se encuentran exclusivamente en el domonio de las eubacterias, donde también se pueden encontrar enzimas del otro grupo (Tabla II). En los eucariontes no se han descrito enzimas accesorias capaces de bombear iones, aunque se han caracterizado relativamente pocas cadenas respiratorias.

Los elementos respiratorios accesorios más comunes en los eucariontes son la NADH deshidrogenasa alterna (NDH-2) y la oxidasa alterna (AOX) (Figura 4). A continuación se describen con profundidad las características de estas dos enzimas respiratorias.

Organismo	Enzima respiratoria accesoria		Referencia
	Bombea iones	No bombea iones	
Eubacterias			
Synechocystis sp	bd quinol oxidasa, ARTO	AOX, NDH-2	53
Bacillus stearothermophilus	caa$_3$ cit c oxidasa, b(o/a)$_3$ cit c oxidasa, bd quinol oxidasa	NDH-2	54
Escherichia coli	bd quinol oxidasa, bo$_3$ quinol oxidasa, fumarato redutasa, quinol oxidasa III, nitrato reductasa,	D- aminoácido deshidrogenasa, glucosa oxidasa formato redutasa, Hidrogenasa, D-L lactato deshidrogenasa , NDH-2, Piruvato oxidasa	55
Arqueobacterias			
Acicianus ambivalens	caa$_3$ cyt c oxidasa	NDH-2	56
Methanosarcina sp.	HHDS oxidasa F$_{420}$H$_2$DH, NiFe hidrogenase		57
Eucariontes			
Arabidopsis thaliana		AOX, NDH-2, NADPH deshidrogenasa	58-59
Neurospora crassa		AOX, NDH-2, NADPH deshidrogenasa	60
Tripanosoma brucei		AOX, NDH-2	61

Tabla II. Distribución de las enzimas accesorias en los tres dominios taxonómicos.

REVIEW

Alternative NADH dehydrogenase: structure, evolution and physiological role

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Short title: Alternative NADH dehydrogenase

Título: La NADH deshidrogenasa alterna: estructura, evolución y papel fisiológico.

Abbreviations: NDH-2; Alternative NADH dehydrogenase, ND2e; external isoform of alternative NADH dehydrogenase, ND2i; internal isoform of alternative NADH dehydrogenase.

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Abstract

Many eukaryotic and prokaryotic species present a rotenone-insensitive NADH respiration, supported by alternative NADH dehydrogenase (NDH-2). In contrast with complex I, this enzyme is not associated with the generation of an electrochemical gradient, and therefore does not participate directly in the synthesis of ATP. In spite of its negative contribution to energy production, it is found in many taxonomic lineages, pointing to an important role in cell physiology, which may include the protection against oxidative damage or as a mitochondrial redox shuttle. Additionally, NDH-2 shows a rapid induction response at the expression level, so the cell can adjust the metabolic fluxes in a short time interval.

NDH-2 is composed by a single polypeptide, with FAD or FMN as prosthetic group. Its simplicity is the basis for gene therapy of complex I hereditary defects, and some advances have been done in this respect. However, many structural, catalytic, and regulatory aspects of this enzyme are unknown, including its three-dimensional structure, substrate binding sites, subcellular traffic pathways, and membrane association. The aim of this review is to make a critical analysis of these and other issues.

Keywords: Electron transport chain, NDH-2, protein evolution, reactive oxygen species, redox shuttle.

Resumen

Una gran cantidad de especies eucariontes y procariontes muestran una actividad respiratoria dependiente de NADH, que es insensible a rotenona, debido a la presencia de la NADH deshidrogenasa alterna (NDH-2). A diferencia del complejo I respiratorio, ésta enzima no es capaz de generar una diferencia de potencial electroquímico de iones a través de las membranas biológicas, por que lo no participa directamente en la síntesis de ATP. A pesar que no contribuye en la producción de energía, es una enzima ampliamente distribuida a lo largo de diversas ramas biológicas, lo cual sugiere que tiene un papel fisiológico importante, como puede ser la protección contra el estrés oxidativo o como parte de los sistemas de lanzadera mitocondrial de equivalentes reductores. Además, la NDH-2 muestra una respuesta rápida a nivel de la expresión genética, por lo que la célula puede reajustar el metabolismo rápidamente dependiendo de sus necesidades.

La NDH-2 está compuesta por un solo péptido, con FAD o FMN como grupos prostéticos. La terapia génica con la NDH-2, encaminada a remediar los defectos hereditarios del complejo I, está basada en su sencillez estructural, y avances importantes se han hecho a este respecto. A pesar de esto, hay una gran cantidad de información que es desconocida, incluyendo su estructura tridimensional, los sitios de unión de los sustratos, sus vías de tráfico subcelular, el mecanismo de asociación con la membrana, entre otras. El objetivo de esta revisión es esclarecer algunos de estos aspectos.

Palabras clave: Cadena transportadora de electrones, NDH-2, evolución de las proteínas, especies reactivas de oxígeno, lanzadera redox.

I. Introduction

Oxidative phosphorylation is the biochemical process that couples electron transport and oxygen consumption to ATP synthesis. The electrons, provided by diverse reactions in glycolysis, tricarboxylic acid cycle, β oxidation, and other pathways, flow through a series of redox centers into the final acceptor, O_2. In this process, a proton electrochemical gradient is generated across the mitochondrial or plasma membranes, which is used by a great number of endergonic reactions, such as the synthesis of ATP or the transport of metabolites and proteins across the membranes. The oxidative phosphorylation system is composed by two modules: the respiratory chain and the phosphorylation system. The respiratory chain is in turn formed by four protein complexes that drive the electron transference (complexes I-IV), but only three of these (I, III and IV) generate the electrochemical proton gradient. The phosphorylation system is composed by adenine nucleotide translocase, phosphate carrier and complex V, which catalyze ATP synthesis, obtaining the energy through the controlled dissipation of the proton gradient.

The four complexes are arranged in a unit that can be defined as the basic respiratory chain, which is widely distributed. In addition, a great variety of organisms such as eubacteria [1, 2], archeobacteria [3], protists, fungi and plants [4, 5], and even animals like bivalves, helminthes and polychaetes [6, 7], present accessory elements that branch the electron transfer pathway. Some of the accessory enzymes pump ions through membranes, while others do not. Among this last group stand out, by their ubiquity in eukaryotes, the alternative NADH dehydrogenase (NDH-2) and the alternative oxidase (AOX) (Figure 1). The physiological role of NDH-2 and AOX has been extensively debated, because of their negative contribution to ATP synthesis, although some hypotheses suggest that they could be part of the cellular redox regulatory systems or work as overflow mechanisms.

In spite of the unknown three-dimensional structure of AOX, the residues involved in the binding of cofactor [8-10] and substrate [11] were identified, and the mechanisms used for the regulation of its activity in several organisms have been described [12, 13]. For NDH-2, there are fewer studies covering some gross details of its structure, regulation and physiologic role [4]. In this work, the recent advances in the NDH-2 field are analyzed in a critical way.
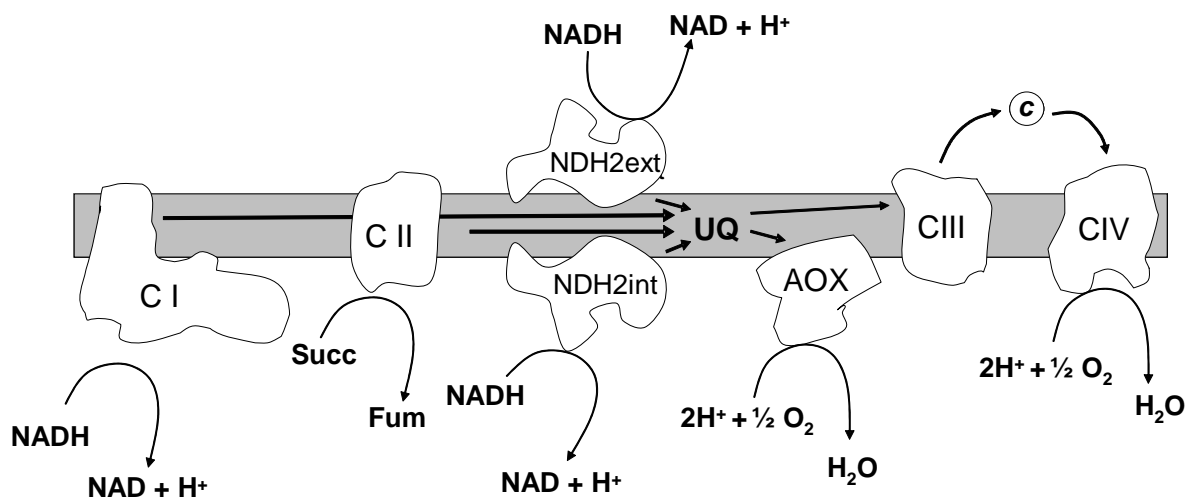
Figure 1. Mitochondrial electron transport chain. AOX; alternative oxidase, CI; rotenone sensitive NADH: ubiquinone oxidoreductase, CII; succinate: ubiquinone oxidoreductase, CIII; ubiquinol: citochrome c oxidoreductase, CIV; citochrome c oxidase, ND2e; external isoform of alternative NADH dehydrogenase, ND2i; internal isoform of alternative NADH dehydrogenase.

II. Types of NADH dehydrogenases

The enzymes that catalyze the electron transference from NADH to ubiquinone are classified in three classes (Table I). Class I are the proton pumping NADH dehydrogenases, also known as the complex I- related enzymes. These proteins are located in the inner mitochondrial membrane in eukaryotes, and in plasma membrane in bacteria, and from a structural point of view show a peripheral and a membranal domain, and are composed of 13-14 subunits in bacterial species, to more than 45 subunits in mammals [14], with molecular masses ranging from 550 to 850 kDa. The prosthetic groups of class I dehydrogenases are up to nine Fe-S clusters and a non-covalently bound FMN molecule, and can be inhibited by tightly-bound inhibitors like rotenone, amytal or piericidine. Class I dehydrogenases show a wide distribution, so there are few organisms where this class is absent, such as some ascomycetous fungus [15], amitochondriate protists (for example *Giardia lamblia*, *Entamoeba histolytica*) [16-19] and some bacterial species [3, 20].

	Type of NADH dehydrogenase		
	I	II	III
Localization	Plasma or mitochondrial membrane	Plasma or mitochondrial membrane	Plasma membrane
Pumps	H^+	Do not pump ions	Na^+
Cofactors/ type of binding	FMN/ non covalent 2 [2Fe-2S] 6-8 [4Fe-4S]	FAD or FMN / non covalent FMN/ covalent	FAD/ non covalent 2 FMN/ covalent [2Fe-2S]
Subunits	from 13 to 45	1	6
Inhibitors	Rotenone, Piericidine A, Capsaicine, Acetogenines, Pyridaben, Fenpyroximate	HDQ, flavone and hydroxilated derivatives	Ag^+, HQNO, Korormycin
Distribution	Except in ascomycetous fungi and extermophillic bacteria	Except animals and some bacteria	Halophylic and pathogenic bacteria

Table I. Characteristics of the three classes of NADH: ubiquinone oxidoreductases. Modified form "Complex I Home Page". [http://www.scripps.edu/mem/biochem/CI/ overview.html].

Class II, or alternative NADH dehydrogenase group (NDH-2), includes proteins composed by a single polypeptide, with a molecular mass from 30 to 70 kDa. Until recently, it was accepted that the prosthetic group found in Class II was exclusively a FAD molecule bound by non-covalent forces, but as discussed below there are some exceptions. In contrast to class I and III NADH dehydrogenases, NDH-2 activity is not associated with the generation of a difference in electrochemical potential across the membrane, and in consequence, does not participate directly in ATP synthesis. It is located in the plasma membrane in bacteria and in the mitochondrial inner membrane in eukaryotes, where depending of its orientation, external or internal isoforms can be found. NDH-2 seems to be a peripheral enzyme, but the mechanism by which the protein associates with the membrane is not known. It is insensitive to class I and III inhibitors, but is inhibited by flavone and some of its hydroxylated derivatives [4].

Class III dehydrogenases couple the oxidation of NADH with the movement of sodium ions across the plasma membrane. They are not sensitive to rotenone or flavone, but are inhibited by silver ions and by 2-heptyl-4-hydroxiquinoline N-oxide (HQNO). Their distribution is restricted to hallophylic and some pathogenic bacteria [21-23].

A fourth type of NADH: ubiquinone oxidoreductases includes the NAD(P)H diaphorase group. Diaphorases are soluble cytosolic enzymes that catalyze the electron transference from NADH to several types of quinoloid compunds, but their natural substrate seems to be menadione [24]. Unlike the other classes, their function is not related to oxidative phosphorylation, but to vitamin K1 biosynthesis [25] or in the handling of semiquinone radicals [26]. In contrast with the other types, diaphorases have similar catalytic efficiencies with NADH and NADPH [27].

Among the three classes of NADH dehydrogenases involved in oxidative phosphorylation, NDH-2 is one of the most frequently found in living organisms, and its fluctuations in activity correlate with a large number of factors, such as cell growth rate, nitrogen or carbon source, and temperature. Furthermore, while the activity of complex I in mammalian mitochondria is the only pathway by which the electrons from NADH enter into the respiratory chain, in fungal, plant and microbial species the NDH-2 have a major role in cell life.

III. NDH-2 structure

The three-dimensional structure of NDH-2 is unknown. However, several secondary structure prediction algorithms show that NDH-2 is an α/β protein, consistent with the group of flavoenzymes to which is related [4].

III. i. Substrate binding site

III. i. i. NADH and FAD binding sites

The enzymes that bind nucleotides, such as ADP, ATP, NAD, NADH or FAD, belong to very diverse groups, including dehydrogenases, transferases, ligases, and many others. In spite of the very low sequence similarity found between these groups, the folding

pattern of the regions involved in nucleotide binding is very similar, showing a βαβ fold as the basic unit [28, 29], as found in the Rossman fold ($\beta_1\alpha_1\beta_2\alpha_2\beta_3\beta_4\beta_5\beta_6$). Three glycine residues are also well conserved in this structure, in an array GxGxxG, which was identified by Wierenga as part of the footprint of the nucleotide binding proteins [28]. The glycine residues lay in the interface region between the first β strand and the α helix, forming either a loop or the amino terminus of the helix, and have both structural and binding functions. Their small size allows the β1 strand and the α helix to accommodate in a proper position, and to participate in the binding of the pyrophosphate moiety of the nucleotide, through the union of a structurally conserved water molecule, which interacts with one of the two prochiral oxygen atoms of the nucleotide β phosphate. The protein derived ligands of the water molecule are the peptide backbone of the first (or second) and the third glycine residue, and a highly variable ligand, which in the Rossman fold is an acidic residue at the end of the β4 strand [30].

In spite of their large sequence divergence, the alignment of several NDH-2 sequences shows some conserved regions, two of which are proposed to be the FAD and NADH binding sites [4], since they present the mentioned array of glycine residues and a predicted secondary structure of the type βαβ (Figure 2A and 2B). In the NDH-2 family, the first and second glycine residues are conserved in the two nucleotide binding sites, but the third glycine can be substituted by other small residues, such as alanine and serine, as described in other nucleotide binding proteins [31]. In both motifs, an acidic residue is located at the end of the second β strand, which seems to be essential for the binding of the adenosine moiety, through the establishment of hydrogen bonds between the acid residue with two hydroxyl groups in the ribose ring [28]. However, in some of the NDH-2 sequences, the acid residue can be substituted by glutamine, and interestingly, all the eukaryotic NDH-2 show a serine in this position, suggesting that either the carboxyamine group from glutamine and the hydroxyl group from serine can bind the ribose residue, or that in the binding of the nucleotide there are other complementary, and presumably, more important interactions.

A.

```
Esccol  ------------MTTPLKKIVIVGGGAGGLEMATQLGRKKKAKITLVDRNHSHLWKPLLHEVATGSL
Anavar  -VEALDNNPPHKVVIVGGGGFGGLYAAKAL-AKAKVDVTLIDKRNFHLFQPLLIYQVATGTL
Nospun  -VNSLDNNPPHKVVIVGGGGFGGLYAAKTL-AKAAVNVTLIDKRNFHLFQPLIYQVATGAL
Myctub  -----QPPRRHRVVIIGSGFGGLNAAKKL-KRADVDIKLIDRTTHHLFQPLIYQVATGII
Aratha  -TYSWPDNKRPRVCILGGGFGGLYTALRLEEDKPQVVLVDQSERFVFKPMLYELLSGEV
Triery  -QGEKPSNKITKICILGSGFGGLLYTALYLINKHKHCNCEVIILDQHDHLIVFTPLIYEVITDEL
Nocfar  -VPQTQAASRRRVVIVGSGFAGFTCAKHLCGEDEVEIVLSPLDYMLYFLLIPLFPDVAGGLI
Strcoe  ------MSRPRIVIVGAGFAGYRTARTLSTRHQADITILNPTDYFLYLPLIPLPQVAAGIL
Neucra  -PQVEPDPSKK--TLVVLGTGWGSVSFLKKLD-TEHYNVTVIVSPRNYFLFTPLIYQVATGTI
Yarlip  -DQLPADPSKK--TLVVLGSGWGSVSFLKKLD-TSNYNVIVVSPRNYFLFTPLLPSCPTGTI
Saccer  -VIDPQHSDKPNVLILGSGWGAISFLKHID-TKKYNVVSIISPRSYFAFTPLLPSAPVGTV
Soltub  -EQNQPESKKKRVVVLGTGWGGTSFLKDVD-ISSYDVQVVSPRNYFAFTPLLPSVTCGTV
Aciamb  -------MTKVLVLG-GRFGALTAAYTLVGGSKADVKVINKSRFSYFRPALPHVAIGVR
Sulsol  ----MRLRSMTKVLVLG-ARFGGLSAAYALAGAGNKAEIKVINNTRFSYFRPALPHVATGVI
```

B.

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Esccol  NLGANGK----VNIAIVGGGATGVELSAELHNAVKQLHSYGYKGLTNEALNVTLVEAGERIL
Anavar  EKDPAKRRAWLIFVVVGGGPTGVELAGAIAELAYKTLKEDFRNIDTTETQVILIEGLDRVL
Nospun  ETDPEKRRAWLITFVIVGGGPTGVELAGAIAELANQTLIKEDFRNIDTSEAKILLIEGLDRIL
Myctub  SSDPERRAKLITFTVVGAGPTGVEMAGQIAELAEHTLKGAFRHIDSTKARVIILDAAPAVL
Aratha  DGS------AIKVAAVGCGYAGVELAAATISERLQD-----------RGIVQSINVSKNII
Triery  ------LIRVSIVGGGPSGVELAGKIADRLGR--------------RGEIRLIERGKEIL
Nocfar  DDPEVRLRARRTFVVVGAS-AGTELIVAQLRALADTYADRRGFAREEIR--FLLDTAERVM
Strcoe  DDR-AECAARCTFVVVGAG-TGTEVAAHGALMYTDAQVRRHPMRTGMRPRWMLLDVAPRVM
Neucra  KGSQEEIDRLLHHMVVGGGGPTGVEFAAELQDFFEDDLRKWIP-DIRDDFKVTLVEALPNVL
Yarlip  E-DPEVRLRILHTVVVGGGGPTGVEAAAGELQDYVHQDLRKFLP-ALAEEVQIHLVEALPIVL
Saccer  LPGDPERRRLLSIVVVGGGGPTGVEAAAGELQDYVHQDLRKFLP-SVKDFVKITVIQSGDHIL
Soltub  PGSEEERRTNLHFVIVGGGGPTGVEFEMSLMLHGYFKKKGMLDKVHVTVFSPGE-YL
Aciamb  IGSGPFYQGPKPKVPENFVPADSACEGPVFEMSIMLHGYFRHIDSTSEAKILLIEGLDRIL
Sulsol  IGSGLFYQGPKPKVPENYVPADAACEGPIFEMSIMLSGYFKKKGMLNKVKFVVFSPGE-YL
```

C.

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Esccol  --YIEADLMWAAGIKAPDFL
Anavar  VKQIASKTVIWAAGVKASAMG
Nospun  VKEIASKTVIWAAGVKASAMG
Myctub  VRRIESACKVWSAGVSASRLG
Aratha  SQIIEADIVIWWTVGAKP-LLT
Triery  SVIILTDLVIWTAGTQ----V
Nocfar  --VVPTHTVAWVTGVTGAPIL
Strcoe  --TVDTRTLVWCVGVRPDPLV
Neucra  KITLPYGLIWATGNAVRPVV
Yarlip  TLEMPYGTLIWATGNTVRPVV
Saccer  EETIPYGTLLIWATGNKARPVI
Soltub  HVEVPYGMVVWSTGVGTRPFV
Aciamb  --TIPADITILLPPYTGNPAL
Sulsol  --TIKADLTILIPPYTGNPAL
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D.

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Ustmay  YALGDDASTIDTRLIDQLYDFVD----RY--DKDKRLSYSEFETFAQAIRRKFP-----IASKHFIKLREVFDQYRYPQVDLYLKNKHLLEAKDLFRDSEGNEREEVDIEGFKLALSHVDSQMKSLPATAQ
Neucra  YAIGDCSTIQNNVADHIITFLRNLAWKHGKDPESLELHFSDWRDVAQQIKKRFP-----QATAHIKRLDKLFEEYRYPQVELYLKSKHMRHINDLLADSEGNARKEVDIEAFKLALSEADSQMKTLPATAQ
Aratha  YAVGDCASIAQRKILGDIANIF-----KAADADNSGTLTMEELGVVDDIIVRYPQVDLIIRYPQVELYLKNKHIILEAKDLFRDSEGNEREEVDIEGFKIALSHVDSQMKSLPATAQ
Soltub  YALGDCASVDQHKVMEDISTIF----EAADKDDSGTLSVEEFRDVLEDIIIIRYPQVDLYLKNKHLLEAKDLFRDSEGNEREEVDIEGFKLALSHVDSQMKSLPATAQ
```

Figure 2. NDH-2 sequence alignment. A) Putative FAD binding site, B) Putative NADH binding site, C) Conserved tryptophan residue, D) EF-hand motifs. In dashed letters are shown the conserved residues involved in nucleotide (GxGxxG and E/D/Q) (A and B) binding, the conserved tryptophan residue (C) or the Ca++ binding residues (D). Aciamb; *Acidianus ambivalens* (AJ489504), Anavar; *Anabaena variabilis* (ZP_00161526.2), Aratha; *Arabidopsis thaliana* (NM_120955); Esccol; *Escherichia coli* (NP_415627); Myctub; *Mycobacterium tuberculosis* (NP_336359.1), Nospun; *Nostoc punctiforme* (ZP_00106299.1), Neucra; *Neurospora crassa* (XP_331372.1), Nocfar; *Nocardia farsinica* (YP_118634.1), Soltub; *Solanum tuberosum* (AJ2455862), Strcoe; *Streptomyces coelicolor* (NP_630578.1), Saccer; *Saccharomyces cerevisiae* (NP_013586.1), Sulsol; *Sulfolobus solfataricus* (ArNP_343636), Triery; *Trichodesmium erythraeum* (ZP_00326982.1), Yarlip; *Yarrowia lipolytica* (XP_505856.1).

The crystallographic evidence of NDH-2- related enzymes [33], such as lipoamide dehydrogenase and glutathione reductase, shows that FAD is found in the first nucleotide binding site –located very close to the amino terminus– and NADH is found in the second site –located in the medial section of the protein, so in the NDH-2 family this array could be conserved [32]. Indeed, this organization is found in the three- dimensional modelling of *Escherichia coli* NDH-2 [34]. Interestingly, the model showed that the redox carrier groups of the FAD and NADH molecules (isoalloxazine and nicotinamide rings) are oriented in parallel, and lie very close (< 3 Å), allowing the direct electron transfer. It is possible that NADH transfer the electrons to the *re* face of isoalloxazine ring and the ubiquinone accepts the electrons on the *si* face, a mechanism described for other enzymes in this family [35]. The model also illustrates that this protein presents a large and open hydrophylic pocket through which NADH can accede to its binding site.

Until recently, it was assumed that NDH-2 present exclusively a FAD molecule as cofactor, bound by weak interactions. However, the trypanosomatid NDH-2 has a non-covalently bound FMN molecule [36], and the NDH-2 from thermophylic archeobacteria (*Acidianus ambivalens* and *Sulfolobus metallicus*) contains a covalently bound FMN molecule, which possibly represents a strategy to increase the stability of the protein at temperatures above 80 °C [3]. Strikingly, the latter enzymes do not exhibit the second nucleotide binding motif, instead they show a histidine residue, which could be involved in the covalent binding of the flavin [20].

III. i.ii Quinone binding site

Fisher and Rich [11] identidied that many mitochondria and chloroplast proteins display an ubiquinone binding motif, showing the array aliphatic-X_3-H-X_{2-3}-T/S/L. In the NDH-2 family this motif is absent or reminiscent. Instead, this family shows a conserved tryptophan residue (Figure 2C) that could be part of the ubiquinone binding site [37]. In this context, it is known that the interaction of photosystem I with phylloquinone is mediated by a face to face contact (π-π) of a single conserved tryptophan and the ubiquinone rings [38]. However, in the three- dimensional model of NDH-2, the localization of the tryptophan is

close (6 Å) to the adenine ring of NADH [34], hence the tryptophan possibly interacts hydrophobically with the adenine moiety, as reported in other cases [39]. Alternatively, Schmid and Gerloff propose that a histidine residue might be part of the quinone binding site [34]. However, this residue is not conserved; therefore further work is required to clarify this point.

III. ii. Divalent cation binding sites

III. ii. i. Calcium

It is recognized that calcium ions stimulate the external rotenone- insensitive NADH- dependent respiration in fungal and plant mitochondria, in particular in the organisms that show a isoform of NDH-2 (CaND2) that contains a segment close to the carboxy terminus of the protein, with two regions showing high similarity to Ca^{++} binding EF-hand motifs [40, 41] (Figure 2D). In plants, the motif closer to the amino terminus contains the six ligand residues required for calcium binding, and the second motif lacks some of the ligand groups, so it is proposed to be nonfunctional [42]. In the case of the ascomycetous specie *Neurospora crassa*, the second motif contains the six ligand groups, while the first does not [43]. It is possible that during the evolution of the CaND2 isoform, the ancestral type gene (possibly external) recombined with some other protein and acquired the two EF-hand motifs in a single step, and subsequently the divergent evolution of fungi and plant CaNDH2 led to the loss of function of the first or the second motif.

III. ii. ii. Cooper

One of the first attempts to characterize the NDH-2 was performed with the *E. coli* enzyme. In the original work the authors stressed that the NDH-2 copurified with a NADH-dependent cupric reductase [104]. Some years latter, Rapisarda *et al.*[44] suggested that the *E. coli* NDH-2 was capable to reduce both the ubiquinone and Cu^{++}, in view that this enzyme presents a CxxC array, similar to the heavy metal chelating section of some proteins (as cytochrome c). Indeed, the experimental evidence showed that after its purification, the

enzyme presented an equimolar amount of Cu^{++}, and the fluorometric mesurements confirmed the presence of a Cu-thiolate complex, in a water-hidden conformation, which is exposed by the addition of both substrates. Interestingly, after the reduction of Cu^{++} to Cu^{+}, the Cu^{+} is not easily released from the protein, which points to important physiological implications in cooper homeostasis (see below) [44].

III. iii. Oligomeric form

Some reports indicate that the purified NDH-2 from different sources is active as a dimer [36], although, in the mitochondria the NDH-2 also associates with other proteins, forming large aggregates [45-47]. However, the relationship between the oligomerization state with activity is unknown, but it could be related with NADH or quinone channeling. In contrast, the archeobacterial NDH-2 [3] and the external isoform of NDH-2 from *Ustilago maydis* [47] can be active as monomers. However, the predominant oligomeric forms *in situ* are unknown. On the other hand, some organisms such as *S. cerevisiae* o *N. crassa* contain multiple NDH-2 isoforms (Table II), but the association to produce heteroligomers has not been evaluated.

| Organism | Isoform | | | Ref. |
	ND2e	ND2i	CaND2	
Protists				
Acantamoeba castellanii	Yes	Yes	Yes	95
Disctiostelium discoideum	Yes	No	No	96
Plasmodium yoelli	Yes	No	No	97
Tripanosoma brucei	No	Yes	No	98
Fungi				
Neurospora crassa	Yes	Yes	Yes	101, 102
Sacharomyces cerevisiae	Yes (2)	Yes	No	74
Ustilago maydis	Yes	?	Yes	47
Plants				
Arabidopsis thaliana	No	Yes	Yes	59
Oryza sativa	No	Yes	Yes	59
Solanum tuberosum	No	Yes	Yes	42

Table II. NDH-2 isoform distribution in eukaryotes.

III. iv. Membrane Interaction

Regarding to the association of NDH-2 with biological membranes few studies have been done. Some algorithms predict from 1 to 3 transmembranal α helices [37, 44]. However, a detailed analysis of the putative transmembrane segments reveals the presence of several proline and/ or positively charged residues that might generate an enormous energy barrier, and impede the transmembranal localization of these sections.

The experimental evidence indicates that NDH-2 could be a peripheral protein, in view that it can be solubilized by mild sonication or in the presence of low detergent and/or high salt concentrations [37, 42, 48, 49]. The carboxy terminus of the protein was proposed as the membrane association domain, because it is enriched with short hydrophobic regions, predicted as α helices, which could interact horizontally with the membrane. Indeed, in the *E. coli* NDH-2, two short amphipathic positively charged α helices (Arg390-Ala406 and Gly409-Arg424) has been recognized (by modelling) as candidates for the membrane interaction sites [34]. In fact, the proteolytic removal of the carboxy terminus of *Solanum tuberosum* ND2i prevents the membrane association [4]. Additionally, a region in the *E. coli* NDH-2 was considered as the membrane binding motif, due to a weak similarity with a segment of *S-* mandelate dehydrogenase involved in the interaction with the membrane [50]. Given that this region is not conserved, it is unlikely that it is responsible for the membrane localization in all the members of the NDH-2 family, although it is possible that in the *E. coli* enzyme it further stabilizes the anchoring.

Interestingly, the heterologous expression of eukaryotic NDH-2 in bacteria reveals that NDH-2 is bound to the plasma membrane [42, 51], which indicates that the membrane association take place spontaneously, without a specialized protein sorting machinery –a requirement for the use of NDH-2 in gene therapy (see below).

The case of CaND2 could be more interesting, because Ca^{++} seems to stabilize the membrane attachment [42, 48]. For example, the *S. tuberosum* CaND2 is easily released from the membrane in EGTA containing media, but if Ca^{++} is present, the enzyme remains bound, even in a medium with a high ionic strength [42]. The regulatory aspects of Ca^{++} mediated attachment are discussed in section (VIII).

IV. Mitocondrial Import

The genes of eukaryotic NDH-2 are codified in the cell nucleus [52]. Thus, after the protein synthesis in the cytosol, the NDH-2 is directed into mitochondria, mediated by a signal sequence in the amino terminus of 20-80 amino acids that adopts an amphipathic helix conformation with positive net charge [53], a characteristic of mitochondrial target sequences [54].

The import pathways of internal and external isoforms are not completely characterized, but it is clear that they present some differences. In the case of ND2i, the process resemble the import of the nuclear components of the classic respiratory complexes, with the participation of the outer membrane (TOM) and inner membrane (TIM) translocase complexes to internalize the protein, in a voltage dependent way, and the subsequent cleaving of the signal peptide by the matrix peptidase [37, 42, 55]. On the other hand, the import pathway for ND2e is not evolutionary conserved, since the translocation of this isoform in fungi and plants differs considerably, in particular regarding to the processing of the signal peptide. For example, while in the external isoform of *S. tuberosum* the signal peptide is not processed [42], in the *N. crassa* ND2e the signal peptide is cleaved from the protein [56], suggesting that in some cases the protein –or some part of it– might cross the mitochondrial inner membrane, allowing the matrix peptidase to process the amino terminus. Analogous to the import pathway of the internal isoform, the import of the external isoform also depends on the transmembrane potential [42, 56].

Another interesting, but poorly understood issue, concerns to nature of the signal that determines the submitochondrial fate of the NDH-2 isoforms. It may be speculated that an internal sequence is responsible for the intermembrane localization of the ND2e. However, when the signal peptide of *Yarrowia lipolytica* ND2e is replaced with the signal sequence of the NUAM subunit of the complex I, the former external isoform, was redirected to the mitochondrial matrix and becomes internal [57]. Although it is evident that this process is determined by the N-terminus signal peptide, the specific section involved in this decision process is unknown, due to the high similitude of the signal peptides of internal and external isoforms.

V. NDH-2 evolution

NDH-2 shares a great similitude with several members of the NAD(P)H: disulphide oxidoreductase family, such as the cytosolic enzymes glutathione reductase, NADH oxidase and lipoamide dehydrogenase (LADH) [4]. However, the similitude is greater in the first 2/3 of the protein, indicating that the carboxy- terminus of the NDH-2 arose later in evolution, eliciting the acquisition of new features, as membrane association and/ or ubiquinone binding [4].

The origin of NDH-2 in eukaryotes is attributed to the genetic material from the archaean pre-eukaryote, since the α-proteobacterial specie *Rickettsia prowasekii*, the closest bacterial specie to mitochondria [58], does not present a NDH-2 gene [4], although the protein is present in other eubacteria [59], including other α-proteobacterial species. It is possible that the lack of NDH-2 in *R. prowasekii* is the consequence of genome simplification, a strategy used by many parasites, specially the intracellular species [58, 60]. Kerscher [4] suggested that the first NDH-2 isoform in eukaryotes was the external, because some species, as *Y. lipolytica* [61] and probably *Ustilago maydis* [47], present only this isoform, but scenarios where the internal isoform was lost cannot be discarded.

In mono and dicotyledon plants, as *Oryza sativa* and *Arabidopsis thaliana*, three classes of NDH-2 have been described: A, B and C. In the studied cases, class A[58] and C[90] codify for internal isoforms and are photoinducible. Meanwhile, class B is external and presents the Ca^{++} binding motifs [59]. A recent report indicates that class C groups within a cyanobacterial clade, an indication that plants acquired class C through the endosymbiotic process that led to chloroplast acquisition [59].

In a recent work, Melo *et al*. [20] proposed other classification scheme. They compared the sequences of bacterial, archaeal and eukaryotic NDH-2, and described three major gruops: A, B and C. Group A includes the NDH-2 enzymes that show the two nucleotide binding motifs (many bacterial, eukaryotic and some archaeal enzymes). In addition to these motifs, group B contains the Ca^{++} binding sites (plant and fungal enzymes). Finally, group C is composed by the enzymes that present only the first nucleotide binding motif −from thermoacidophillic archaea. It is worth to note that these

classification schemes were proposed considering only gross details, and a better classification, based on evolutionary evidence, is required.

VI. Enzyme Kinetics

VI.i Substrate specifity

NDH-2 catalyzes the electron transfer from NADH to ubiquinone in a ping pong kinetic mechanism, with NADH as the first substrate [62]. It is specific for the electron donor, so catalyzes the reaction at appreciable rates only with NADH [63-65], while complex I can accept NADH, NADPH and deamino-NADH. In fact, a strategy to determine complex I activity in the presence of NDH-2, is the determination of deamino-NADH oxidation rate [61, 66].

In addition to the rotenone-insensitive NADH respiration, mitochondria from fungi and plants show a NADPH- dependent respiratory activity catalyzed by an unidentified enzyme. The evidence indicates that the NADH and the NADPH respiratory activities are performed by two independent molecular entities, since the NADPH dehydrogenase activity does not correlate with the variations in the NADH dependent activity, and both have different optimal pH values and different susceptibilities to thiol specific and FAD reactive reagents [67]. In fact, the presence of the acidic residue at the end of the second β-strand of the nucleotide binding motif should interfere with the binding of the NADPH molecule, and in fact, in the enzymes that bind NADPH, the acidic residue is replaced by a positively charged residue, as arginine [29].

NDH-2 is less specific for the electron acceptors, and can use a large variety of naturally occurring and synthetic compounds, including DCPIP (2,6-diclorophenol-indophenol), ferricyanide and diverse quinones. The preference for acceptors depends on the source of the enzyme. For example, *S. cerevisiae* ND2i [37] and *Y. lipolytica* ND2e [68] show higher activities with hydrophylic quinones, while *E. coli* NDH-2 displays higher rates with hydrophobic quinones, such as n-decyl ubiquinone [65].

Our group reported that the *S. cerevisiae* NDI shows substrate inhibition by both NADH and DCPIP (or ubiquinone) [62]. The inhibition by the varied substrate is higher at

low concentrations of the fixed one, and as the concentration of fixed substrate rises the inhibition decreases. The mechanism of substrate inhibition was not studied, but the evidence points to a mutually exclusive binding of NADH and DCPIP$_{ox}$ to the oxidized and reduced form of the enzyme, respectively, in a way in which ternary complexes cannot form (Figure 3). If substrate inhibition follows this mechanism, it would be expected that both products, NAD$^+$ and DCPIP$_{red}$, should inhibit the enzyme. However, the inhibition by NAD$^+$ was not observed, indicating that release of this product could be an irreversible step. This proposal opens an interesting possibility, in which the enzyme can be inhibited at elevated concentrations of NADH when the quinone pool is highly reduced (low ubiquinone), slowing down the production of reactive oxygen species in the electron transport chain (see below).
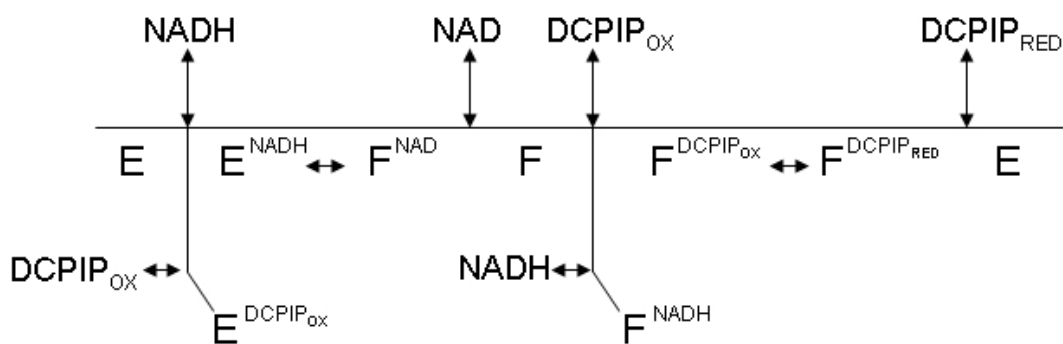
Figure 3. Kinetic mechanism of NDH-2, including a proposal for substrate inhibition. DCPIP$_{red}$, reduced DCPIP; DCPIP$_{ox}$, oxidized DCPIP; E, free oxidized enzyme; F, free reduced enzyme.

VI.ii NDH-2 inhibitors

Few inhibitors of NDH-2 have been described, and flavone is the most widely used [4, 62], with a Ki of 5-7 μM [62]. Unfortunately, the interpretation of data (especially in mitochondrial preparations or cellular extracts) is complicated due to its relatively low specificity [69-73]. Additionally, it is a mixed partial type inhibitor (at saturating concentrations nearly 20 % of the activity remains) [62]. On the other hand, platanetin (6-dimethilalil-3,5,7,8- tetrahydroxiflavone), a hydroxylated derivative of flavone, is another

inhibitor with an $IC_{50} = 2$ μM (flavone IC_{50} varies between 20-95 μM) [4], but there are problems with its permeability through biological membranes. HDQ (1-hydroxy-2-dodecyl-4 quinolone) was recently described as new inhibitor of NDH-2 [68], with a very low Ki (200 nM), but further studies are needed to clarify if this is a tightly- bound inhibitor or not. HDQ exerted a mixed inhibition against both NADH and ubiquinone, raising questions about the catalytic mechanism of this enzyme, because for a ping pong mechanism the expected inhibition patterns would be uncompetitive against NADH and competitive against ubiquinone. It is important to note that the mixed inhibition observed with ubiquinone and NADH is consistent with the model shown in Figure 5, because HDQ will be able to interact with the E and F forms of the enzyme, so the competitive component is the result of HDQ competing with ubiquinone (both binding to the F form), and the uncompetitive component arises from the binding of HDQ to E. Some problems still need to be solved before the use of HDQ as a specific inhibitor of NDH-2. As mentioned by the authors, HDQ inhibits complex I with at least 10 times lesser efficiency than NDH-2, but other ubiquinone/ ubiquinol binding proteins, such as AOX, complex III or succinate dehydrogenase were not tested. Moreover, it is not clear if this inhibitor is total or partial, since 80-90% of the activity remains in the presence of an inhibitor concentration of 50 Ki (10 μM).

VII. NDH-2 metabolic role

VII. i. ND2e as a mitochondrial redox shuttle

An active production of redox equivalents is the result of both the catabolic reactions of the cell, as well as of the biosynthetic processes. The electrons, produced by the cell metabolism, are incorporated mainly into the NAD^+ and FAD coenzymes, and in order to achieve a steady state metabolic flux the cell requires mechanisms that guarantee the reoxidation of the reduced coenzymes. In aerobic cells, one of the major functions of the mitochondria is precisely the oxidation of cytosolic NADH, mediated by a series of multienzymatic systems, known as redox shuttles. In mammals, the most important are the aspartate- glutamate and the glycerol 3-phosphate shuttles, and in other organisms a large

diversity of these systems is found, such as the acetaldehyde- ethanol and malate-oxaloacetate shuttles, along with the ND2e (Figure 6).
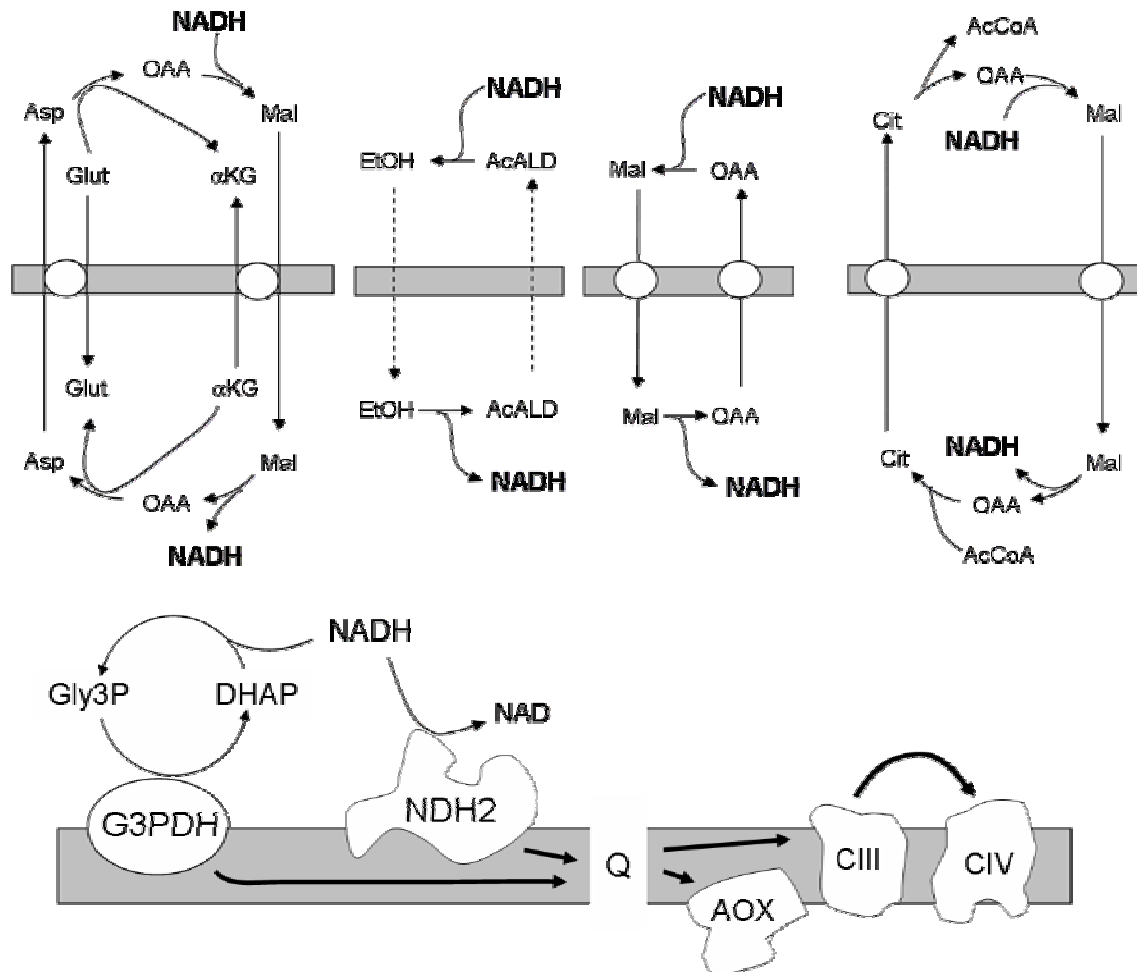
Figure 4. Mitochondrial redox shuttle systems. Up left, glycerol 3-phosphate shuttle. Middle left, ND2e. Bottom left, acetaldehyde- ethanol shuttle. Bottom center, malate-oxaloacetate shuttle. Up right, fatty acid shuttle. Middle right, aspartate- malate shuttle. αKG, α- ketoglutarate; AcALD, acetaldehyde; AcCoA, acetyl- coenzyme A; Asp, aspartate; Cit, citrate; DHAP, dihydroxyacetone phosphate; EtOH, ethanol; G3P, glycerol 3-phosphate; Glut, glutamate; Mal, malate; OAA, oxaloacetate; Pi, inorganic phosphate.

The ND2e system is particularly interesting since this enzyme represents an efficient mechanism through which the electrons of the cytosolic NADH enter directly into the mitochondrial electron transport chain, without the participation of any other

intermediate [74]. In contrast, the other shuttles show appreciable rates only when the concentration of the intermediates (malate, citrate, glycerol 3-phosphate, etc.) reach the milimolar range, compared with the low cytosolic concentrations of NADH (1-10 μM). The requirement for high intermediary concentrations could be a disadvantage, considering that in free living organisms the metabolic machinery is under constant readaptation, due to the changing environment, so the intermediary concentration could have large variations. Thus, the ND2e will allow the oxidation of cytosolic NADH independently of other metabolic pathways, such as glycolysis and tricarboxylic acid cycle, assuring a rapid metabolic response to nutrient availability fluctuations.

In *S. cerevisiae* the NDH2ext seems to be the major pathway for cytosolic NADH oxidation, since the growth in the presence of respiratory substrates is diminished in ΔNDE1 and ΔNDE1 ΔNDE2 strains, but as the growth is not completely impaired, and as the cells did not acquire the petite phenotipe (which appears when the mitochondrial metabolism is absent), the other shuttles must be active [75]. The *S. cerevisiae* strains lacking some components of the other shuttles, such as the glycerol 3-phosphate dehydrogenase, did not show any growth alterations [76], supporting the idea that the external NDH-2 is the major shuttle in this organism. In addition, the regulatory mechanisms futher confirms the main role of ND2e, because the glycerol 3-phosphate oxidation can be "inhibited" in the presence of NADH, possibly by a physical contact between the ND2e and the mitochondrial glycerol 3-phosphate dehydrogenase [77].

Alternatively, in plant tissues the main shuttle mechanism, at low concentrations of NADH (<2 μM), is the malate-oxaloatetate shuttle [78]. However, as the NADH concentration increases, the activity of ND2e attains comparable rates. In this example, the ND2e take part as a mechanism by which the mitochondrial metabolism is able to adapt the oxidative phosphorylation to higher cytosolic redox states, which may be achieved in a variety of circumstances, for example, during the onset of photosynthesis [79].

VII.ii. The physiologic role of ND2i

Ascertain the function of ND2i has been difficult, mainly because this enzyme compete with complex I for both substrates, producing a negative effect on the energetic

state of the cell. However, the presence of the internal isoform could be advantageous in some circumstances, as: a) in a fluctuating medium or under stress conditions, since the protein induction time might be lower for NDH-2 than for complex I, which is composed by more than 40 subunits, whose assembly pass through several steps and depends on the expression and regulation of mitochondrial and nuclear genes; b) when the activity of complex I is compromised, for example under low sulfur or iron availability, a particularly important condition for parasite organisms, because a large production of iron chelators is triggered by the immune response [80]; and c) when cell economy is not in risk and rapid growth is required, as at the start of the colonization process. In example, in *Torulopsis utilis* [81] and *N. crassa* [82] it was shown that NDH-2 is more active throughout the exponential phase of growth, and in the stationary phase is replaced by complex I.

Due to the large diversity of NDH-2 isoforms, the metabolic adaptations of the cell and the presence of redundant pathways (as complex I or other redox shuttles), the physiologic relevance of NDH-2 is reliant on the particular organism. In example, in *N. crassa* the deletion of complex I produced viable cells, because its loss is compensated by the ND2i [83]. In *Y. lipolytica* there is no effect on the growth in the ΔND2e strain, while defects in complex I are lethal [61]. In the fungal genus *Saccharomyces*, where complex I is absent, its role is fully taken by ND2i. Indeed, yeast strains lacking this enzyme do not grow in respiratory substrates as pyruvate or acetate [84].

VII.iii. Prevention of oxidative damage

Approximately 1-5 % of the electrons flowing through the respiratory chain contribute to the generation of superoxide anion and other reactive oxygen species (ROS) [85]. The main sources of this production are the complex I and III, due to the occurrence of a highly reactive, but relatively stabilized semiquinone ion in their catalytic cycles, which easily react with oxygen. One of the major roles of the alternative components of the electron transport chain is the maintaining of the ubiquinone/ ubiquinol pool in an oxidized state, preventing the over-reduction of the pool, and in turn decreasing the life time of the semiquinone, and in consequence lowering the generation of ROS. Besides, the NDH-2 does not seem to form a semiquinone radical, probably because the electron transfer

reaction proceeds in pairs [86], thus, it provides a pathway through which the electrons enter into the respiratory chain, bypassing the production of ROS by complex I,

Some reports indicate that NDH-2 and AOX are more resistant to reactive oxygen species than complex I or III [5]. This resistance could be another advantage for pathogen organisms; supporting the function of the mitochondrial metabolism during the infection, withstanding the hypersensibility response of plants and the immune response of animals, which involve an active production of oxygen reactive species.

VII. iv. Additional functions

The plant cell metabolism produces large amounts of glycine during photorespiration that must be oxidized, even when the energy state of the cell is high. In this condition the function of the classic respiratory pathways must be impaired, but as the alternative components are not limited by the proton electrochemical gradient and are not adenylate restricted, it is evident that glycine oxidation depends on the activity of NDH-2 and AOX [87]. In the same way, it is possible that the synthesis of amino acids and lipids in high cell energy charge conditions depends on the alternative elements, because their activity would decrease the NADH/ NAD$^+$ ratio, and allow the Krebs cycle to accomplish its anaplerotic roles [88].

In contrast, in the cyanobacteria *Synecchocystis spp.*, NDH-2 is probably involved in a signaling pathway, but neither in respiration or in the maintenance of the phylloquinone redox state. At low light intensities the ΔNDH-2 strains grew as well as wild type strain, but in contrast with the wild type, the ΔNDH-2 strain can grow at high illumination conditions. Although the authors suggest that the NDH-2 participates in a signaling pathway, probably sensing the phyloquinone redox state [89], any direct evidence was not shown.

On the other hand, the *E. coli* NDH-2 catalyzes the reduction of Cu^{++} to Cu$^+$, but the reduced cooper ion remains bound to the enzyme. Some authors suggest that the NDH-2 has a role in copper intracellular transport and metabolism, as a metallochaperonin, delivering Cu$^+$ to a non- identified protein, responsible for its further metabolism [44].

Otherwise, if free Cu^+ is released to the cytosol, the oxidative damage, mediated by the Fenton reaction, would increase greatly,

VIII. Regulation

The stimulation by Ca^{++} of the external NADH respiration in plant and fungi mitochondria [40, 41] led to the proposal that this cation directly activates the CaNDH2. However, this interpretation should be taken with care, since Ca^{++} could activate several other steps in the respiratory pathway, and unfortunately, the effect of Ca^{++} on purified CaNDH2 has not been determined. Nevertheless, some studies showed that Ca^{++} did not stimulate the NDH-2 activity at fixed saturating concentrations of both substrates [48, 49]. However, in these studies the identity of the NDH-2 isoform was not shown, so it is possible that these results were generated with the Ca^{++} insensitive isoforms. On the other hand, it is possible that Ca^{++} activation is observed only when the enzyme is bound to the membrane. Indeed, some authors suggest that the activation mechanism is mediated by the facilitation of membrane-protein interactions [42], resulting in an increase in the enzyme availability on the membrane surface, and consequently, in an enlargement in the *Vmax* of the NADH-dependent respiratory activity. In fact, Ca^{++} activation was found at NADH concentrations three orders of magnitude (>1 mM) higher than the *Km* (4-10 μM) [62], which is consistent with the increase in the *Vmax*.

IX. Gene therapy

Several human diseases are associated with complex I defects, as Leber´s hereditary optical neuropathy and possibly diabetes [91]. In the Parkinson syndrome a participation of complex I defects was also reported. In fact, the treatment with complex I inhibitors, such as rotenone or MPP^+ (1-methil-4-phenilpyridinium ion), promotes the development of Parkinson-like disease in primates and rats. Parkinson syndrome is also associated with the exposition to insecticides and herbicides, as pyridaben and fenpyroximate, both inhibitors of complex I [92].

As part of an integral therapy against these diseases, lays the treatment of complex I defects. One alternative is the use of synthetic compounds to chemically oxidize the

intracellular NADH, but unfortunately they also react with other cellular components, and more important, they cannot be targeted to a specific organ or tissue. On the other hand, gene therapy with the *S. cerevisiae* internal NDH-2 gene is more rational and straightforward, and in fact is even better than the replacement of defective genes with the wild type genes. In a documented case, in which the complex I activity was absent due to a single missense mutation (where lysine was replaced by arginine), the transformation of cells with the wild type subunit recovered only 40% of the activity, suggesting that in the transformed cells complex I was a mixed population of the mutant and the wild type proteins [91]. Therefore, to recover 100% of the activity is essential to introduce the wild type subunit and delete the mutant one, a procedure that seems unviable. On the other hand, gene treatment with *S. cerevisiae* NDI gene bypasses this problem with additional benefits, as the low generation of oxygen radicals.

Takao Yagi´s group successfully transformed (with mechanical techniques) proliferative mammalian cells from diverse sources [91, 93, 94]. They also report that the recombinant protein is active and localized in the mitochondrial compartment, and did not affect the expression of the endogenous complex I. As expected, the transformed cell mitochondria showed decreased ADP/O ratios with substrates as glutamate plus malate, and can grow in the presence of complex I inhibitors.

An encouraging result from this group is the transformation of human brain non-proliferative cells with a non- immunogenic parvovirus (adeno-associated virus). The transformed cells did not show alterations in cell differentiation nor in other parameters [92]. Nonetheless, gene therapy of complex I diseases is still far away in humans, because it is not known whether the transformation with the NDH-2 will rise an autoimmune response by the recognition of the transformed tissue as non-self.

X. Conclusion

In spite of the more than three decades of research on the NDH-2, there are many issues still unexplored. New knowledge should be generated for rational design of organisms or for its use in gene therapy.

XI. Acknowledments

XII, Bibliography

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REVIEW

Structure and Function of Alternative Oxidase

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Abbreviations: AOX; alternative oxidase

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Abstract

Alternative oxidase (AOX) is a respiratory enzyme that catalyzes a terminal oxidase reaction at the level of the ubiquinol pool, similar to the activity of the cytochrome pathway (complex III and IV). Nevertheless, the AOX reaction is not coupled with the generation of an electrochemical potential difference of protons across the mitochondrial membrane, and consequently does not participate in ATP synthesis. Its activity confers a cyanide or antimicyn resistant respiration, because it is not inhibited by the cytochrome pathway inhibitors. It is found in many eukaryotic species including plants, fungi, protists and even some animals. However, its contribution to cell metabolism is still unresolved, because of its energy wasting potential.

Some advances have been made on its three- dimensional structure and catalytic mechanism, but other issues remain as open questions. Here we offer an update on the advances made about AOX, including an analysis of its mechanisms of regulation, contrasting the behaviour of the plant enzymes with the fungal/ protist enzymes. Additionally, a method to estimate its contribution to respiration in intact cells or tissues is described.

Key words: alternative oxidase, respiratory chain, metabolism, reactive oxygen species.

Resumen

La oxidasa alterna (AOX) es una enzima respiratoria que cataliza una reacción de oxidasa terminal a nivel de la poza de ubiquinol, de manera similar a la actividad de la vía citocrómica (compuesta por los complejos respiratorios III y IV). Sin embargo, en la reacción catalizada por la AOX no se genera una diferencia de potencial electroquímico de protones a través de la membrana mitocondrial interna, por lo que esta enzima no participa en la síntesis de ATP. Su presencia le brinda a la célula una respiración resistente a cianuro o antimicina, ya que no es sensible a los inhibidores de la vía citocrómica. Se encuentra ampliamente distribuida en organismos eucariontes incluyendo plantas, hongos, protistas e incluso en algunos animales. A pesar de su ubicuidad su contribución al metabolismo celular se encuentra en discusión, debido a su incapacidad para aprovechar la energía liberada por la reacción redox que cataliza y transformarla en energía útil.

Aunque se han hecho algunos avances para conocer su estructura tridimensional y mecanismo catalítico muchas de sus características son desconocidas. En esta revision se muestra una actualización de los estudios recientes sobre la AOX, incluyendo un análisis de sus mecanismos de regulación, donde se contrasta el comportamiento de la AOX de plantas y la de los hongos y protistas. Además se describe un método para estimar su contribución a la respiración de células intactas o tejidos.

Palabras clave: cadena respiratoria, especies reactivas de oxígeno, metabolismo, oxidasa alterna.

I. **Introduction**

In vertebrate animals, the mitochondrial oxidation pathway of dihydroubiquinone (QH_2) by the electron transport chain involve two widely distributed enzymes, complex III (a) and complex IV (b), also known as the cytochrome pathway (a+b) [1-3].

$$QH_2 + 2\ citc_{ox} + 2\ H_i^+ \rightarrow Q + 2\ citc_{red} + 2\ H_e^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.... \quad (a)$$

$$4\ citc_{red} + O_2 + 4\ H_i^+ \rightarrow 4\ citc_{ox} + 2\ H_2O + 4\ H_e^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (b)$$

$$2\ QH_2 + O_2 + 6\ H_i^+ \rightarrow 2\ Q + 2\ H_2O + 6\ H_e^+ \quad \ldots\ldots\ldots..\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (a + b)$$
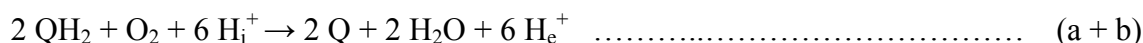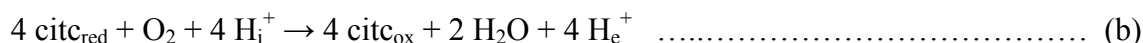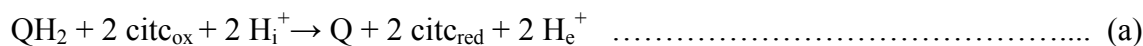
Many organisms present other accessory enzymes that catalyze a terminal oxidase reaction in parallel to the cytochrome pathway. Among these accessory terminal oxidases stands out an enzyme not found in many of the well studied mitochondria (from vertebrates), the alternative oxidase (AOX) [2,3] (c, Fig1), which is the focus of this review.

$$2\ QH_2 + O_2 \rightarrow 2\ Q + 2\ H_2O \quad \ldots....\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.. \quad (c)$$
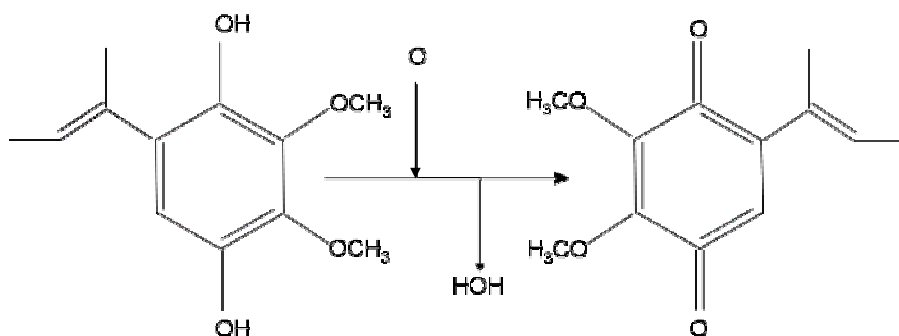
Fig 1. AOX catalyzed half reaction, in which a single dihydroubiquinone molecule and ½ O_2 (O) are shown, rendering as products ubiquinone and a water molecule.

A diagnostic characteristic of the AOX- containing mitochondria is the resistance against inhibitors of the cytochrome pathway (such as cyanide, azide, antimycin, stigmatellin,

methoxyacrylates, among others), and the nearly specific inhibition by phenolic antagonists, as salicylhydroxamic acid (SHAM) and n-alkil gallates (Fig 2) [3].

Salicylhydroxamic acid
(SHAM)

N-Octyl Gallate
(nOg)
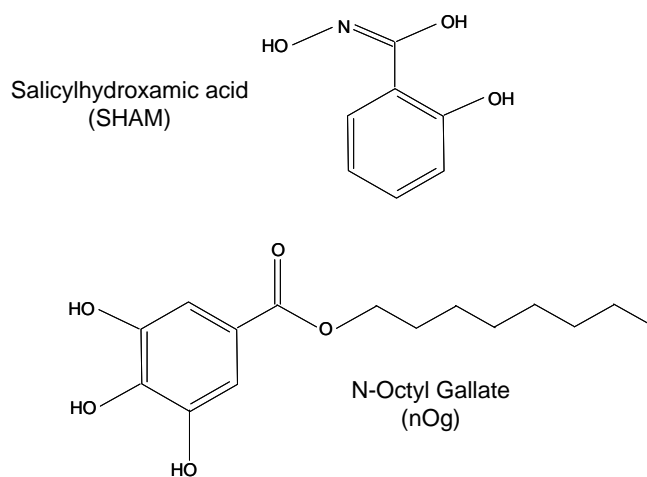
Fig 2. Phenolic antagonists acting as AOX inhinbitors. SHAM (N,2- dihydroxybenzamide or o-hydroxybenzohydroxamic) and nOg (n-octyl ester of 3,4,5-trihydroxibenzoic acid) [7,8].

The presence of cyanide insensitive respiration was observed in many eukaryotic organisms, including all plants studied [3, 4], many fungi [1, 2], many protists [2], and even some animals [5]. However, the physiological role of AOX has been debated along the years, since its activity is not coupled with the generation of a proton electrochemical potential difference across the mitochondrial inner membrane [6], and bypasses two phosphorylation sites, which potentially reduce the ATP yield to 1/3. In this review we analyzed some of the recent findings concerning AOX structure and regulation.

II. AOX Structure

Although neither spectroscopic, nor crystallographic data are yet available to provide any direct evidence of AOX three-dimensional structure, the current view is that AOX is a non-heme di-iron carboxylate enzyme, as originally proposed by Siedow *et al.* [9], who identified two conserved E-XX-H motifs (Fig 3), similar to those found in methane monooxygenase. Additionally, it has been shown that the AOX from *Pichia anomala* [10] and *Trypanosoma brucei* [11] require iron ions for their catalytic activity, because iron chelators inhibit either its expression or activity.

```
Trybru    TCRWLFDTFSLYRFG------------------SITESKVISRCLFLETVAGVPGMVGG
Neucra    ICRWATDIATGIRPEQQVDKHHPTT---ATSADKPLTEAQWLVRFIFLESIAGVPGMVAG
Aratha    KADITIDLKKHHVPTTFLDRIAYWTVKSLRWPTDLFFQRRYGCRAMMLETVAAVPGMVGG
Orysat    TADTSIDLTKHHVPKTLLDKIAYWTVKSLRFPTDIFFQRRYGCRAMMLETVAAVPGMVGG
Novaro    MIPPFIDLSVHHKPGGLSDRIAFGFTKALRWCADTFFAERYGHRAVVLETVAAVPGMVGA
Ustmay    AAATTTSQSSTTSPTPTATKVVNQTLQEMRAKGLSFGPDGWLNRMIFLESIAGVPGMVAA
Crypar    LEEVNNVQKTHLCPNGFKDKMSYYLFDLLTRYKKGHNEYQWCRRIIFLETVAGVPGMVGA
```

Helix 1

```
Trybru    MLRHLSSLRYMTRDKGWINTLLVEAENERMHLMTFIELR-QPGLPLRVSIIITQAIMYLF
Neucra    MLRHLHSLRRLKRDNGWIETLLEESYNERMHLLTFMKMC-EPGLLMKTLILGAQGVFFNA
Aratha    MLLHCKSLRRFEQSGGWIKALLEEAENERMHLMTFMEVA-KPKWYERALVITVQGVFFNA
Orysat    MLLHLRSLRRFEQSGGWIRTLLEEAENERMHLMTFMEVA-NPKWYERALVITVQGVFFNA
Novaro    TINHLACLRRMCDDKGWIKTLMDEAENERMHLMTFIEIS-KPTLFERAVIMGVQWVFYLF
Ustmay    TCRHLQSLRLMRRDKGWIHTMLEDAENERMHLLVALHLSGKPGLIARTFVLLAQGVFYNF
Crypar    MLRHFSSLRKMKRDNGWIHTLLEEAENERMHLLISLQLINKPSILTRVSVIGTQFAFLIF
```

Helix 2   HH

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Trybru    LLVAYVISPRFVHRFVGYLEEEAVITYTGVMRAIDEGRLRPTKN---DVPEVARVYWNLS
Neucra    MFLSYLISPKITHRFVGYLEEEAVHTYTRCIREIEEGHLPKWSDEKFEIPEMAVRYWRMP
Aratha    YFLGYLISPKFAHRMVGYLEEEAIHSYTEFLKELDKGNI-----ENVPAPAIAIDYWRLP
Orysat    YFLGYLLSPKFAHRVVGYLEEEAIHSYTEFLKDLEAGKI-----DNVPAPAIAIDYWRLP
Novaro    FFGLYLVSPKTAHRVVGYFEEEAVISYTHYLAEIDQGRS-----ANVPAPAIAKRYWGLP
Ustmay    FFIFYLLSPRVAHRFVGVLEEEAVLTYSLILEDLKEGRLP--EWEDVPAPEIAKQYWQLG
Crypar    YTVFYIISPKYSHRFVGYLEEEAVSTYTHLIEEIDKGLLP--G-FERKAPKFASVYYGLP
```

Helix 3

```
Trybru    KN-ATFRDLINVIRADEAEHRVVNHTFADMHEKRLQNSVNPFVVLKKNPEEMYSNQPSGK
Neucra    EGKRTMKDLIHYIRADEAVHRGVNHTLSNLDQK---EDPNPFVSDYKEGEGGRRPVNPAL
Aratha    AD-ATLRDVVMVVRADEAHHRDVNH-FASDIHYQGRELKEAPAPIGYH------------
Orysat    AN-ATLKDVVTVVRADEAHHRDVNH-FASDIHYQGMELKQTPAPIGYH------------
Novaro    DN-AMLRDVVLVVRADEAEHRDVNHGFANELAGLPVAEPAACPPHHALEPNWKKAA----
Ustmay    DE-AMLVDVIRAIRADEATHRHINHTFASLNSDDPNPFALREPPAKMRAETYGLERDEAL
Crypar    ED-ATIRDLFLAMRRDESHHRDVNHNLADIRLNGE------------------------
```

Helix 4

Figure 3. Sequence alignment of AOX sequences, showing the coordination residues of the di- iron center. Aratha, *Arabidopsis thaliana* (NP_188876); Crypar, *Cryptosporidium parvum* (AAEE01000004); Neucra, *Neurospora crassa* (AAC374); Novaro, *Novosphingobium aromaticivorans* (NZ_AAAV03000002); Orysat, *Oryza sativa* (BAA28772.); Trubru, *Trypanosoma brucei* (AAB46424); Ustmay, *Ustilago maydis* (EAK83685). In black are shown the residues involved in iron coordination. In gray, the residues that can donate electrons in the catalytic cycle.

Two structural models appeared from the initial observation of Siedow *et al.* [9] (Fig 4): a) the oxygen binding di- iron centre is buried in a four helix bundle, and consequently AOX should interact horizontally with the membrane [12], or b) AOX presents two membrane spanning helices connected by an extramitochondrial helix, with the active site inside the mitochondrial matrix [9].
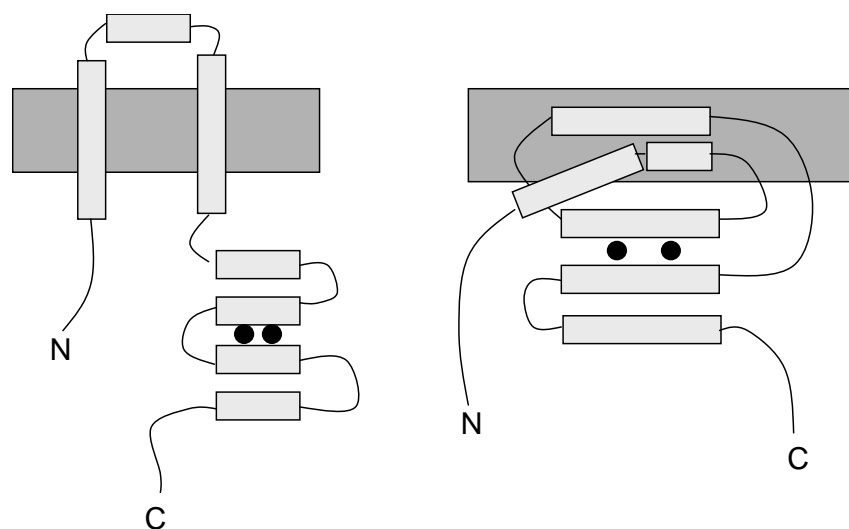
Figure 4. Structural hypotheses about the three-dimensional structure of AOX. Gray rectangles show the helical segments of the protein. Black circles indicate the iron ions bound to the enzyme.

Albury *et al.* [13] realized that a single site- directed mutagenesis experiment should be necessary to test the two hypotheses. They observed that E217 was part of the catalytic domain in the four helix bundle proposal, but was not involved in catalysis in the alternative proposal. The mutagenesis experiment showed that the E217A mutant was a completely inactive enzyme, supporting the view that AOX does not contain any transmembranal domain. It is valuable to stress that the structural considerations, as the length of the helices and the localization of the iron binding sphere, have previoulsy predicted that AOX could be folded in a four helix bundle, as other di- iron–carboxylate enzymes do [12, 14].

The active site of AOX comprises two pairs of antiparallel α helices (Figs 4 and 5), containing six ligand residues for the di- iron centre. Two of these are glutamate residues

located at the more N terminal helix of each helix pair (helices 1 and 3), and the last four are the glutamate and histidine residues found in the E-XX-H motifs, located in the C terminus helix of each helix pair (helices 2 and 4) [12, 15].
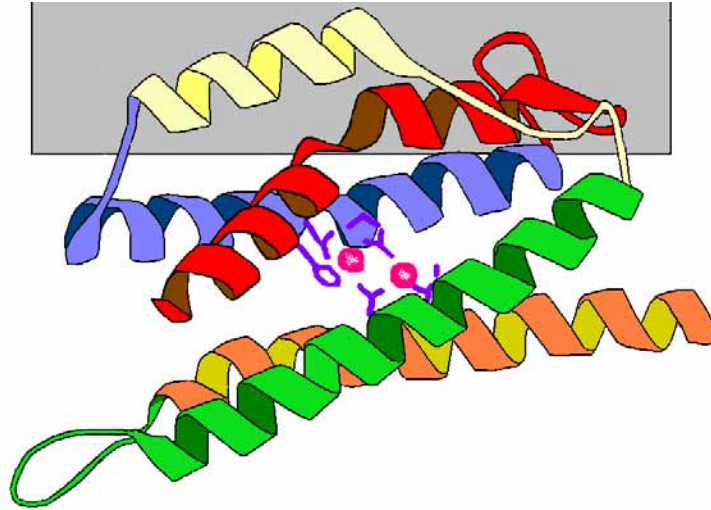
Figure 5. Three- dimensional model of AOX structure. Red, blue, yellow, green and orange helices represent helices 1, 2, HH, 3 and 4, respectively. Modified from Finnegan *et al.* [19].

The identity of the iron ligating glutamate residue in helix 3 has been uncertain, because of the presence of three consecutive glutamate residues (Fig 3). Changes in the N terminal E residue in *Arabidopsis thaliana* AOX eliminated the distinctive EPR signal [16], while changes in the second or third residue in *Trypanosoma brucei* [10, 17] or the third in *Sauromatum guttatum* AOX, abolished the activity [13, 18]. These results imply that species-specific residues are involved in iron coordination. Indeed, *Dictiostelium discoideum* AOX and *Plorococcus* PTOX (Plastid Terminal Oxidase) lack the C terminal residue, *Nostoc* and *Synecococcus* lack all but the N terminal E residue [19], and *A. thaliana* PTOX does not present the middle E residue [14].

In addition to the central four helix bundle, it is now recognized that a fifth helix structure lying between helices 2 and 3 (HH), is present, which along with the C terminal part of helix 1 show hydropathy profiles that indicate that these regions interact with the membrane [12] (Fig 5).

II. i. Ubiquinol binding site

Fisher and Rich [20] identified a quinone binding motif in many chloroplast, bacterial and mitochondrial enzymes. Although some AOX sequences present a variation of this motif in the N- terminus of the protein (with a composition aliphatic $-X_3$-H-X_{2-3} − L/T/S), it is absent in many AOX, remarkably in those from fungi and protists.

To solve this question, Berthold [21] expressed *A. thaliana* AOX mutants in haem deficient *E. coli*, and tested the susceptibility for SHAM inhibition. The mutant strains F259L, M263I, M263V and G347E displayed significant increases in the resistance against SHAM, which suggested that these residues can be part of the quinol binding site. However, the comparative analysis of the available AOX sequences showed that only M236 is conserved.

III. Catalytic mechanism

Two models were initially proposed for the catalytic mechanism of AOX [22, 23]. In both, the enzyme is fully reduced through the simultaneous reaction with the two ubiquinol molecules (Fig 6A), producing a reduced hipoferrous di- iron centre (Fe^1-Fe^1). Once reduced, the enzyme is oxidized in a single step with an oxygen molecule, producing two water molecules. However, it is now evident how difficult would be to accommodate four electrons in a single binuclear di- iron centre [15].

Interestingly, other di- iron-carboxylate proteins, such as stearoyl-ACP Δ^9 –desaturase, methane monooxygenase and ruberithrin are capable to fully reduce oxygen to water as a side reaction [24- 26]. The study of these enzymes have provided a background for the proposal of the catalytic mechanism of AOX, and two models emerged which solved the electron overload problem [14, 15]. The two models are similar but differ essentially in the presence of a peroxide intermediate. Recently, the studies made with methane monooxygenase showed that a diferryl intermediate is formed during the catalytic cycle, both in the oxidase and monooxygenase reaction [26]. This result conducted Affourtit *et al.* [15] to propose an improved catalytic proposal shown in figure 6B. In this new model, the oxidized (diferric) enzyme is reduced to the diferrous state, in a reaction where the first ubiquinol molecule is oxidized. In the ferrous state the AOX is able to interact with

molecular oxygen to produce the enzyme-dioxygen Michaelis complex, which in an unimolecular reaction lead to the formation of the peroxydized enzyme, which protonates twice and releases the first water molecule along with the enzyme in a diferryl state (detected in methane monooxygenase reaction cycle), which reacts with the second ubiquinol molecule, releasing the last two products.

A.   B.
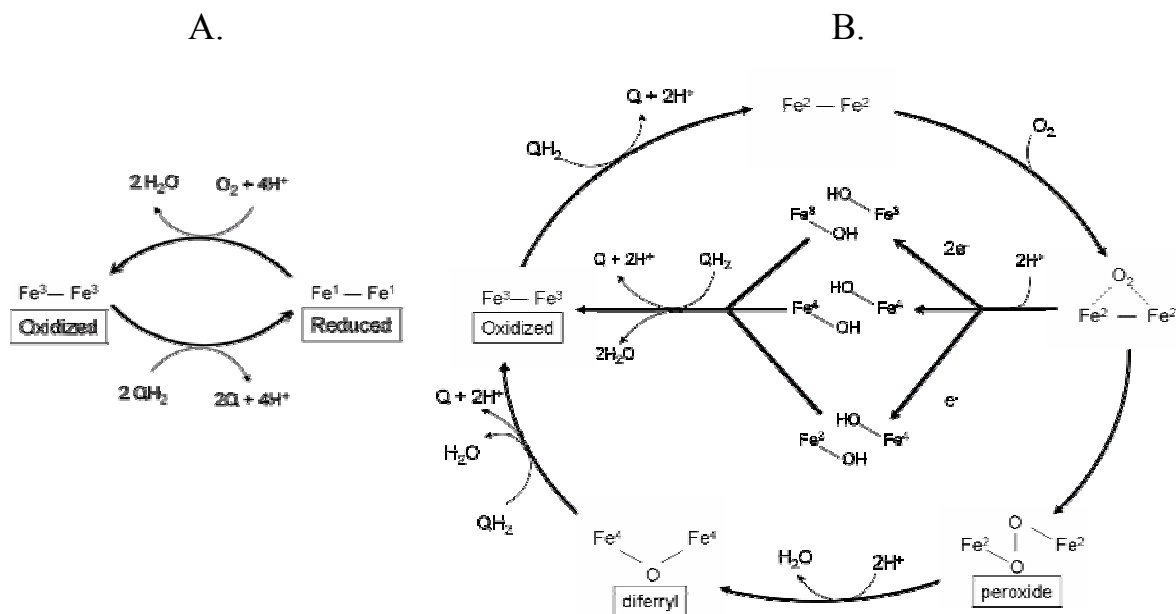
Figure 6. Catalytic mechanisms proposed for the AOX reaction. A) Catalytic model of Siedow *et al.* [22] and Ribas-Carbo [23]. B) Catalytic model of Affourtit [15].

The studies performed with citochrome *c* oxidase indicate that both haem, and probably the non-haem, oxygen metabolizing enzymes reduce the molecular oxygen in a single step [27], releasing the two water molecules simultaneously. Although in this catalytic mechanism, the oxidation of the protein backbone is required. Taking this fact into account, an alternative model was also formulated by Affourtit *et al.* [15], in which the enzyme-dioxygen complex is formed and the oxygen is fully reduced, but incompletely protonated. Subsequently, the enzyme transfers one or two electrons from the peptide backbone, to the molecular oxygen, and recapture the electrons during the oxidation of the second ubiquinol molecule. The authors suggest that the electrons can be taken either from the di- iron centre or from the amino acid residues, producing radical species. Indeed, the mutagenic change

of a conserved tyrosine residue (close to the di- iron centre) to phenylalanine (Y275F), produced a completely inactive enzyme [14]. A tyrosine residue is also absolutely necessary during the catalytic cycle of cytochrome *c* oxidase [27]. Interestingly, Albury *et al.* [13] recorded the signal of a ferric – ferryl species, consitent with the production of a radical in the enzyme backbone. This intermediate is also found in the catalytic cycle of ribonucleotide reductase, another di- iron centre protein [28]. In addition, the analysis of AOX sequences showed a universally conserved tryptophan residue (W206), which could be another internal source of electrons.

IV. AOX evolution

It is known that many of the characterized di- iron carboxylate metalloenzymes catalyze the reduction of dioxygen, as a byproduct, no matter of their large functional diversity. Recently, Gomes *et al.* [26] described that ruberithrin, a member of this protein family, can reduce molecular oxygen, accepting the electrons from NADH probably through a NADH-dependent: ruberithrin reductase. The authors suggest that this enzyme family arose to scavenge the early traces of oxygen and that the oxygen reduction activity is in fact an adaptation of early microorganisms to the rising in oxygen levels, and then the proteins diverged and acquired particular functions, but preserved their ancestral catalytic activity. In anaerobic organisms, other enzymes are responsible for the protection against oxygen, presenting as cofactors iron and cooper ions. However, Cu^+ presents a very low solubility in a reducing environment, a predominat condition in the early atmosphere [29]. Therefore, it is possible that the di- iron carboxylate enzymes indeed appeared very early in evolution, and the other systems appeared only when the atmosphere turn in a far more oxidative state. In eukaryotic organisms, two enzymes of the di- iron carboxylate family are involved in energy metabolism, AOX in mitochondria, and PTOX in chloroplast, sharing a limited, yet significant similitude. In *A. thaliana*, the PTOX catalyzes a terminal oxidase reaction in the chloroplast, and when expressed in *E. coli*, this enzyme confers a cyanide resistant respiration that can be inhibited by alkyl-gallates [30]. Due to its localization it was termed plastid terminal oxidase (PTOX). PTOX is present in many photosynthetic lineages as in

plants, cyanobacteria and algae, and is possibly involved in carothenoid biosynthesis or in chlororespiration,

The evolutionary trends drawn from the analysis of Finnegan *et al.* [19] showed that PTOX and AOX proteins were acquired by the eukaryotes in two independent events. The eukaryotic AOX sequences are more related with the α− proteobacterial AOX (from *Novosphingobium aromaticivorans*) than to other proteins. The PTOX from plants and other photosynthethic eukaryotes are more related to cyanobacterial PTOX. This scenario implies that AOX was acquired by eukaryotes in the endosymbiotic process where the mitochondrion was obtained, from an α− proteobacterial specie. Meanwhile, the plants acquired PTOX in the endosymbiotic event that lead to chloroplast acquisition, from cyanobacterial species.

V. Regulatory mechanisms

The regulatory mechanisms of plant AOX have been deeply investigated. The activity of AOX is considerably increased upon reduction of an intersubunit disulphide bridge [31]. In this form the protein is further activated by α-ketoacids, particularly pyruvate, that seems to react with a reduced cysteine residue, producing a thiohemiacetal [32]. It is possible that the formation of the thiohemiacetal is catalyzed by AOX itself, since pyruvate can activate AOX in inside-out mitochondrial particles or in the solubilized enzyme [35, 36].

The regulatory domain of plant AOX is located in the N- terminal region of the protein, and presents two cysteine residues that are higly, but not universally, conserved in the plant enzymes (C122 and C172, in *Sauromatum guttatum* numbering). Mutagenesis studies revealed that the N- terminal cysteine (C122) is the regulatory residue [33], and interestingly the mutation of C to E yields a constituvely active AOX, resembling the wild type enzyme activated by pyruvate. Subsequent studies showed that the C122S [34] or the C122A [19] mutants were not activated by pyruvate, but were specifically activated by succinate, in contrast to the wild type enzyme that is not stimulated by this metabolic intermediate. The mechanism involved in the physiological reduction of AOX was unclear until recently. It was known that some substrates, such as malate and isocitrate, can indirectly activate AOX, probably through the production of the reducing agent NADPH [1, 3], which in turn increase

the concentration of thioredoxin and gluthathione that could be the natural activators of AOX [31]. In fact, both substances can reduce AOX *in vitro* [36], and it is now clear that some plants contain a mitochondrial type of thioredoxin (thioredoxin h) that specifically activates AOX, which is enzymatically reduced by a NADPH- dependent thioredoxin reductase [37]. Experiments carried out with the Q electrode demonstrated that the AOX activity shows a sigmoidal behaviour as the ubiquinol/ ubiquinone ratio (QH_2/ Q) is modified, showing an appreciable activity only when the redox state of the ubiquinone pool is elevated, while the Cyt pathway presents a nearly linear behavior [38]. The activation by pyruvate seems to be mixed, decreasing the apparent affinity for quinol and increasing the Vmax of the AOX, turning the enzyme more active at low QH_2/ Q ratios [39]. Our group recently reanalyzed the data of Krab [38], and found that the sigmoidal response of AOX can be fitted very well taking into consideration the inhibition by the product (Table 1). Our analysis predicts that a high activity of AOX at low QH2/ Q can be obtained in two different conditions: a) with a 350 fold increase in the Vmax, or b) with a decrease of ten times in the Km value, together with an increase in the product inhibition constant (Ki) by nearly 10 times.

	Km_{QH2}	Ki_Q
	(QH_2/Q+QH_2)	(Q/Q+QH_2)
Cyt pathway	0.05	3
AOX inactive	0.8	0.15
AOX activated	0.07	2

Table 1. Kinetic parameters of *Solanum tuberosum* AOX. The data was taken from Krab [38] and fitted using a single substrate Michaelis- Menten function considering competitive product inhibition.

Interestingly, the activity of AOX is not only a function of the redox status of the ubiquinol pool, the reduced/ oxidized quotient of AOX, or the pyruvate concentration, but also depends on the substrate used, since in plant mitochondria the AOX activity is higher with succinate than with NADH, even though NADH produces a higher reduction of the ubiquinone pool [40].

Contrasting with the plant enzyme, the fungal and protist AOX are monomeric enzymes that are not stimulated by pyruvate nor by the reducing treatment [41]. Instead they are

activated by purine nucleotides, such as ADP, AMP and GMP [41]. The fungal AOX does not present the regulatory domain of plant AOX, where the conseved cysteines are located, which has been suggested as the dimerization site, explaining why fungal enzymes do not form dimers. However, *Gaemamomyces gramminis* AOX is dimeric, raising the possibility that other fungal AOX can be dimers, linked by weak electrostatic forces [33].
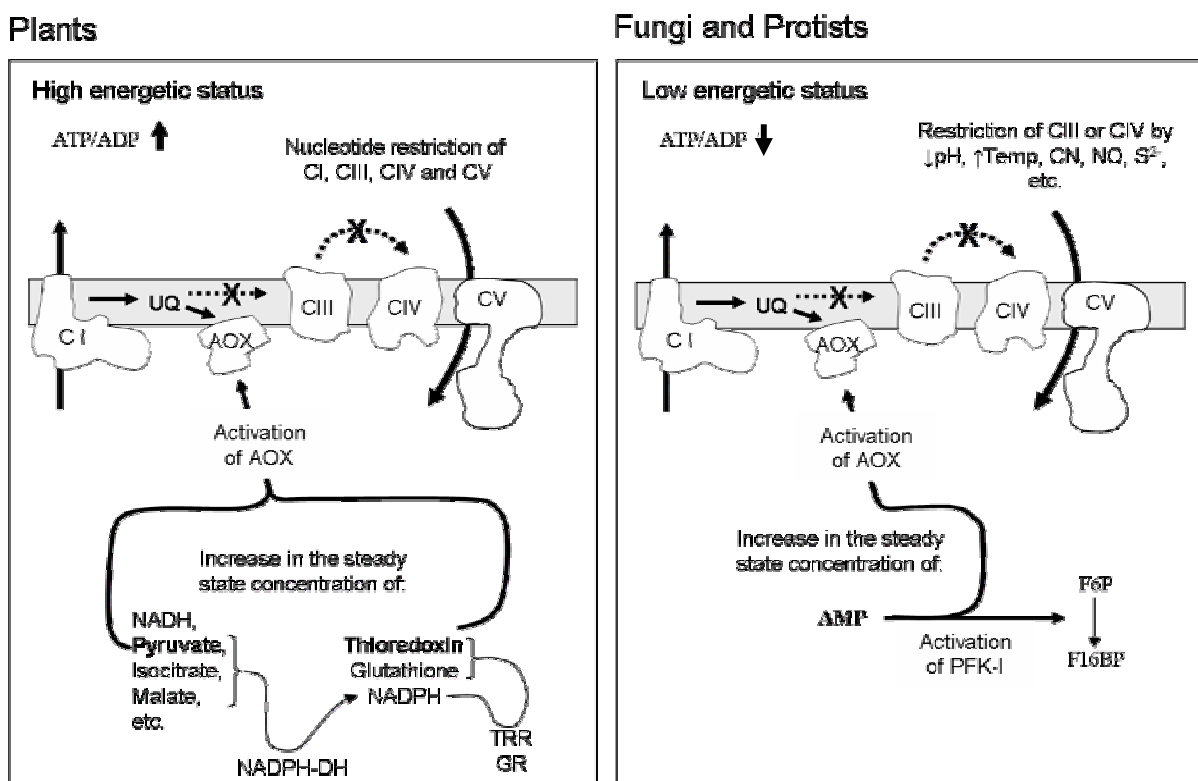
Figure 7. Aspects of the activation of AOX in plants and fungal/ protists species.CI; complex I, CIII; complex III, CIV; complex IV, CV; complex V, GR; glutathione reductase, NADPH-DH; NADP dehydrogenases dependent on isocitrate, malate and pyruvate, TRR; thioredoxin reductase.

The work of our group [64] showed that the regulation of AOX in fungi and plants seems to respond to different metabolic stimulation (Figure 7). In plants, the activation by pyruvate should be present under high energetic conditions. When the energetic status of the cell is high, as during the onset of photosynthesis, the mitochondrial oxidative phosphorylation may be nucleotide restricted, and consequently the levels of ubiquinol and NADH will rise,

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producing an increase in the steady state concentration of some intermediaries, such as pyruvate, malate or isocitrate. As malate and isocitrate are the substrates of some $NADP^+$ dependent dehydrogenases, the steady state concentration of NADPH will also rise, and the concentrations of glutathione and thioredoxin can increase, preventing oxidative stress. In this condition, pyruvate and thioredoxin are able to activate AOX, thus preventing the mitochondrial metabolism to collapse. In agreement with this view, the chlororespiration depends on AOX activity [42].

In fungi and protists species, the activation occurs when a low energetic status is reached. A great number of abiotic factors, as temperature [43, 44, 47], low pH values [45], sulphur ions [5], cooper availability [51], and biotic factors as NO [49-52] and cyanide [53] (both produced actively during the response of organisms against pathogens) or antimycin and stigmatellin (produced by some soil bacterial species [54, 55]), are known to limit or abolish the activity of the Cyt pathway. In these conditions the energetic status of the cell is reduced, and the levels of AMP, produced by adenilate kinase, will rise. These factors in turn will increase the glycolytic flux through PFK-I [54] and the activity of AOX, in a kind of rescue response.

VI. Contribution of AOX to cell and mitochondrial respiration

Bahr and Booner [57] originally proposed that the electrons flowing through the Cyt pathway or through AOX cannot be taken or redirected toward the other pathway. With this assumption, it was apparently easy to determine the contribution of AOX to respiration by simply measuring the SHAM sensitive respiration. However, in many cases the SHAM sensitive respiration was much less (nearly zero) compared to the cyanide resistant respiration. This result suggests that the electrons can be redirected to one or the other pathway, therefore, the contribution of AOX was underestimated by the experimental approach of Bahr and Booner [58].

Two methods were reported to unambiguously measure the contribution of AOX to respiration. The first approximation[59] is based in the difference of affinity toward oxygen[18] (O^{18}) between AOX and the Cyt pathway. As demonstrated, the partitioning of O^{18} favours AOX over the Cyt pathway; thus by measuring the oxygen flow and the production of H_2O^{16} and H_2O^{18} it is possible to determine the activity of AOX. It is worth to note that this

method presents some complications, but the most important are that it is not known whether the partition of O^{18} is constant or can be altered in the intact cell or tissue, and its is assumed that the O^{18} partitioning is independent on the ubiquinone redox state or the activation state of AOX.

W. Jarmuszkiewickz et al. [60] proposed another method for the estimation of AOX contribution to mitochondrial respiration, based on the selective activation of the cytochrome pathway by ADP (in the presence of phosphate and Mg ions). However this method cannot be applied when complex I is active, since its activity respond to the proton electrochemical difference.

Our group recently reported [64] other method to qualitatively estimate the contribution of AOX to cell or tissue respiration, which can be easily developed in many laboratories, although it can only be applied to strictly aerobic organisms. The method is based on a simple hypothesis: if a significant part of the electrons flow through AOX, then the inactivation (inhibition) of the enzyme, should increase the ATP content, at an the extent that depends on the metabolic state of the cell, i.e. the relative activities of the ATP producing and consuming processes. However, it is possible that the increasse in the ATP concentration could decrease the rate of the ATP producing processes, and therefore, upon the inhibition of AOX the respiration may also be decreased. On the other hand, if only a few electrons flow through the AOX, the addition of the AOX inhibitors would not have any effect on the ATP content or on cell respiration. The experimental evidence found in the fungal phytopathogen *Ustilago maydis* showed that the ATP content and the respiratory rate are unaffected by the AOX inhibition [64], which suggests that AOX does not contribute at high extent to cell respiration. However, some points must be clarified prior to the application of this method, such as the relative contribution of oxidative phosphorylation and other pathways to ATP production, because this approximation might be applied to aerobic organisms where the inhibitors of the electron transport chain or the uncouplers have a negative effect on the ATP content.

A more comprehensive method to determine the AOX activity in many metabolic states is the mathematical modelling of the metabolic network. However, a detailed kinetic characterization of all the enzymes in the system is necessary, and as yet the kinetics of AOX has been only partially characterized.

VII. Physiologic role

VII.i. Oxidative stress protection

In aerobic organisms a substantial ammount of the ROS production is the direct result of the mitochondrial metabolism, in particular of the respiratory pathway, since one or two semiquinone ions are formed in the catalytic cycles of complexes I and III. This highly unstable intermediate can react with O_2, producing superoxide, and thus initiate the ROS production cascade. The AOX role in ROS defense seems to be the prevention the ubiquinone pool over- reduction, which decreases the half time of the semiquinone radical and lowers its steady state concentration. In fact, it is very well documented that the activity of AOX correlates inversely with the production of ROS [61].

VII.ii. Metabolic plasticity

As mentioned above, many environmental conditions are known to limit the activity of the cytochrome pathway. Additionally, the biotic interactions are also characterized for the development of strategies to contend against other organisms or to avoid and attack of pathogens. Among these strategies, the active production of inhibitors of the cytochrome pathway is remarkable, such as the methoxyacrylate, antimycin and stigmatellin secretion by soil bacterial species, and the cyanogenesis and NO production in the hypersensitivity response of plants. In these conditions, the AOX activity would allow the intermediary metabolism to proceed, maintaining the activity of the TCA cycle, which depends on the rate of NADH oxidation, and hence the biosynthesis of amino acids and lipids.

VII.iii. Thermogenesis

In addition to the previous roles, the AOX accomplish other functions in some organisms. The flowering tissues of certain plants maintain a temperature 5-7 °C above the environment temperature, which is necessary to volatilize primary amines, used to attract pollinating insects. In these tissues a great activity of AOX is found, and some authors

suggest that this enzyme can contribute to high extent in the heat production. However, it is now clear that other energy dissipating processes have a greater contribution to this process, as the uncoupling protein, some pyrophosphatases and other enzymes [62].

VII.iv. Overflow mechanisms

In some tissues it is recognized that AOX has a major role in cell respiration, in particular under highly energetic conditions, such as during mitochondrial state 4, where Cyt pathway is nucleotide restricted [42]. From this observation arose the notion that AOX can work as an overflow mechanism [63], similar to a valve system, keeping active the mitochondrial metabolism and other processes as chlororespiration, and diminishing the production of ROS. Additionally, the activation of AOX by α-ketoglutarare suggests that the ammonium assimilation could also depend on AOX activity [36].

VIII. Acknowledgements

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Durante la década de los setentas y principios de los ochentas se realizaron estudios para conocer el efecto de ciertos plaguicidas o antibióticos, como la carboxina o el cloranfenicol, sobre el metabolismo mitocondrial de *U. maydis* [14-18]. En estos trabajos se reportó la presencia de una oxidasa terminal insensible a cianuro y antimicina [14-18], y se demostró que un porcentaje significativo de la oxidación del NADH era insensible a rotenona [15,18], lo que sugería la presencia de elementos respiratorios alternos. Sin embargo, los estudios no profundizaron en estos temas y la línea de investigación no tuvo continuidad, por lo que el conocimiento de la energética de este hongo era muy escaso.

Para comenzar a entender el metabolismo energético de *U. maydis*, nos propusimos como primer objetivo el estudio de la organización de la cadena respiratoria. En la siguiente sección se hace una breve descripción de las cadenas respiratorias de los eucariontes, y se aborda con profundidad la estructura y función de los componentes alternos más ubicuos, la oxidasa alterna (AOX) y la NADH deshidrogenasa alterna (NDH-2).

JUSTIFICACIÓN

A pesar de la gran cantidad de información disponible sobre la biología de *Ustilago maydis*, muchas aspectos permanecen sin ser descritos, principalmente su metabolismo energético e intermediario. Desafortunadamente esta falta de estudios no es exclusivo de este hongo, sino de la mayoría de los basidiomicetos. De hecho, hasta la aparición de nuestras aportaciones, se habían publicado sólo cinco trabajos en los que se abordaron someramente algunos aspectos del funcionamiento de la mitocondria de este organismo, mientras que otros procesos, como la glucólisis o el ciclo de Krebs, por mencionar algunos, son totalmente desconocidos.

Nuestro interés principal fue la caracterización de la cadena transportadora de electrones mitocondrial, haciendo énfasis en dos de las principales enzimas accesorias, la oxidasa alterna (AOX) y la NADH deshidrogenasa alterna (NDH-2), y su papel en el funcionamiento celular.

2. OBJETIVOS

"Look at your young men fighting
Look at your women crying
Look at your young men dying
The way they've always done before

Look at the hate we're breeding
Look at the fear we're feeding
Look at the lives we're leading
The way we've always done before

Look at the shoes your filling
Look at the blood we're spilling
Look at the world we're killing
The way we've always done before
Look in the doubt we've wallowed
Look at the leaders we've followed
Look at the lies we've swallowed
I went so numb when I learned to see
And I don't want to hear no more".
Guns and Roses, 1991

Objetivo general

Determinar la función metabólica de las enzimas respiratorias que no participan en la síntesis de ATP en el hongo fitopatógeno *Ustilago maydis*.

Objetivos particulares

1. Caracterizar la organización de la cadena transportadora de electrones mitocondrial de *U. maydis*.

2. Determinar las funciones metabólicas que desempeñan la oxidasa alterna y la NADH deshidrogenasa alterna en *U. maydis*.

3. RESULTADOS

"I went out walking through streets paved with gold
Lifted some stones, saw the skin and bones of a city without a soul
I went out walking under an atomic sky, where the ground won't turn
And the rain it burns like the tears when I said goodbye
Yeah I went with nothing, nothing but the thought of you
I went wandering

I went drifting through the capitals of tin, where men can't walk or freely talk
And sons turn their fathers in
I stopped outside a church house, where the citizens like to sit
They say they want the kingdom, but they don't want God in it

I went out riding
Down that old eight lane
I passed by a thousand signs
Looking for my own name

I went with nothing, but the thought you'd be there too
Looking for you

I went out there in search of experience
To taste and to touch, and to feel as much
As a man can, before he repents

I went out searching looking for one good man
A spirit who would not bend or break, who would sit at his father's right hand
I found on him nothing, nothing but lies and fear
I fought the fear, through pain, blood and tears,
Now I see the sun, finally I am free".
Johnny Cash & U2, 1993

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3.1 La organización de la cadena respiratoria mitocondrial de *Ustilago maydis*

The mitochondrial respiratory chain of *Ustilago maydis*

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Abstract

Ustilago maydis mitochondria contain the four classical components of the electron transport chain (complexes I, II, III, and IV), a glycerol phosphate dehydrogenase, and two alternative elements: an external rotenone-insensitive flavone-sensitive NADH dehydrogenase (NDH-2) and an alternative oxidase (AOX). The external NDH-2 contributes as much as complex I to the NADH-dependent respiratory activity, and is not modulated by Ca^{2+}, a regulatory mechanism described for plant NDH-2, and presumed to be a unique characteristic of the external isozyme. The AOX accounts for the 20% residual respiratory activity after inhibition of complex IV by cyanide. This residual activity depends on growth conditions, since cells grown in the presence of cyanide or antimycin A increase its proportion to about 75% of the uninhibited rate. The effect of AMP, pyruvate and DTT on AOX was studied. The activity of AOX in *U. maydis* cells was sensitive to AMP but not to pyruvate, which agrees with the regulatory characteristics of a fungal AOX. Interestingly, the presence of DTT during cell permeabilisation protected the enzyme against inactivation.

The pathways of quinone reduction and quinol oxidation lack an additive behavior. This is consistent with the competition of the respiratory components of each pathway for the quinol/quinone pool.
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Keywords: Alternative NADH dehydrogenase; Alternative oxidase; Basidiomycete; Digitonin; Electron transport chain; Salicylhydroxamic acid; Permeabilisation; *Ustilago maydis*

1. Introduction

Ustilago maydis is the causal agent of corn smut; infection by this organism produces galls in the fruits of its host plant, filled with teliospores. In many countries this fungus causes a severe damage to crops [1]. In addition, *U. maydis* is related to other phytopathogens (grouped in *Tilletia* and *Ustilago* genus) which infect economically important species such as rice, sugar cane or sorghum [1,2].

For these reasons, *U. maydis* has been the subject of an intense research, especially at the level of its genetic regulation, virulence, development, and relationship with the host [1,3], such that it is considered a model phytopathogen [1–3]. However, the bioenergetics and intermediary metabolism of *U. maydis* are far from understood, and this problem is shared with the great majority of the basidiomycetous species.

One important characteristic of plant and some fungal mitochondria is the presence of alternative components in their respiratory chains, which branch the pathway of electron transfer and are not coupled to ATP synthesis [4–6]. The most ubiquitous and prominent of these enzymes are AOX (quinol oxidase) [4] and NDH-2; the latter catalyzes the same redox reaction as complex I but does not pump protons across the mitochondrial inner membrane [5].

However, other alternative components might be present, such as diverse dehydrogenases and quinol oxidoreductases [7–9]. Hence, the complexity of respiratory chains may vary from very simple linear chains to highly branched ones. *S. cerevisiae* illustrates the first situation, with mitochondria containing no complex I, no AOX, and two NDH-2 embedded in the inner mitochondrial membrane [4]. At the other end, plant mitochondria show a high degree of branching, with several external and internal NAD(P)H dehydrogenases in addition to complex I, and one AOX [10].

The physiological role of alternative respiratory complexes is unclear. Evidence suggests that they are involved in the adaptation of organisms to fluctuating environments. Cold stress, oxidative stress, anaerobic conditions, change in food source or in temperature [11–13] are among the factors that influence the expression or activity of alternative respiratory components. Recently, it has been addressed the question of the function of the external isoform of NDH-2. This enzyme is thought to be the main shuttle of reducing equivalents in *S. cerevisiae* [14,15].

About 30 years ago, Ziogas and Georgopoulos [16], looking for the mechanism of action of carboxin, described in *U. maydis* mitochondria a significant percentage of the respiration resistant to cyanide or antimycin A. They also reported that exogenous NADH stimulates respiratory activity, and that a percentage of this activity was resistant to rotenone. These two resistant activities were increased in the presence of chloramphenicol [17], an inhibitor of mitochondrial protein synthesis, which is known to reduce the activity of classic respiratory complexes. Together, these results suggest the presence of two alternative components, an AOX and an NDH-2.

The aim of this study was the further characterization of the mitochondrial respiratory chain of *U. maydis*, with particular emphasis on the identification and regulation of the alternative components.

2. Materials and methods

2.1. Cell culture

Strain FB$_2$ (a$_2$b$_2$) of wild-type *U. maydis* was used in this study. Saprobium yeast-like monokaryotic cells were grown as previously reported [18] in YPD medium (1% yeast extract, 0.25% bactopeptone, 1% glucose), pH 4.7 at 29 ± 2 °C, under shaking at 250 rpm. The flasks were filled to one quarter of their capacity to prevent anaerobiosis. At 48 h of culture, cells were harvested by centrifugation and washed twice with distilled water. Finally, cells were suspended in KME medium (KCl 120 mM, MOPS 20 mM, EDTA 2 mM, pH 7) in which the experiments with intact and permeabilised cells were performed. Cell density was determined by reading the absorbance at 600 nm (AU_{600}). To study the long-term effect of respiratory inhibitors on the activity of AOX, cells were grown for 24 h at 30 °C, followed by the addition of inhibitor, and harvested after further 24 h of incubation at 30 °C.

2.2. Cell permeabilisation

Plasma membrane permeabilisation was achieved by incubation of *U. maydis* cells (35 AU_{600}/ml) with 20 mg/ml digitonin, for 1–3 min at room temperature, followed by centrifugation in a microfuge. Cells were resuspended in KME medium and placed on ice. To preserve the activity of AOX, permeabilisation and resuspension of cells were carried out in the presence of pyruvate 5 mM [19] and/or DTT 1 mM [20], both activators of plant AOX, and/or AMP 5 mM [21], an activator of the fungal enzyme.

2.3. Mitochondria isolation

U. maydis cells were harvested by centrifugation, washed twice with distilled water, and resuspended in MTE buffer (mannitol 600 mM, Tris–HCl 20 mM, EDTA 1 mM, pH 7.4) at a final ratio of 5 ml/g wet weight. Subsequent steps were carried out in the same buffer at 4 °C. Cells were disrupted with glass beads (in the presence of 1 mM PMSF) and mitochondria were isolated by differential centrifugation. Briefly, cell debris was eliminated by centrifugation at $3000 \times g$ for 10 min. The mitochondrial pellet was obtained by spinning the $3000 \times g$ supernatant for 10 min at $12\,000 \times g$, washed once to eliminate cytosolic contamination, and resuspended with MTE buffer to a final protein concentration of 10–30 mg/ml. Bovine heart and *S. cerevisiae* mitochondria were obtained as described previously [22].

2.4. Oxygen consumption

Respiratory measurements with intact and permeabilised cells were carried out in 1.5 ml of air-saturated KME medium (pH 7) at 25 °C. Oxygen consumption was determined using a Clark-type oxygen electrode.

2.5. Native blue gel electrophoresis

Native blue gel electrophoresis was performed in a 5% to 14% acrylamide gradient gel as described by Schägger and von Jagow [23]. NADH dehydrogenase activities were revealed by incubating the gel for 30–45 min in the dark, in 50 ml of Tris–HCl 20 mM, pH 7.4, with NADH 50 μM and MTT 50 μM. MTT changes its color when reduced, from yellow to violet, and precipitates at the point where the redox reaction occurs.

2.6. Immunoblot

Electrophoresis was carried out with 50 μg of mitochondrial protein, under denaturing and reducing conditions in

10% acrylamide gel. Electrotransfer to PVDF membrane was performed at 100 V for 1 h. Rabbit antiserum against *Clamydomonas reindhartii* AOX was used at 1000-fold dilution. Goat peroxidase-conjugates anti-rabbit IgG antibodies were obtained from Sta. Cruz Labs and used at 10,000-fold dilution. Luminol-based Chemiluminiscence ECL kit was obtained from Amersham Bioscience. Film exposure time was 30 s. The rabbit antiserum against *C. reindhartii* AOX was a gift from Dr. Diego Gonzalez-Halphen from Instituto de Fisiología Celular, UNAM.

3. Results

3.1. The classic respiratory complexes in U. maydis mitochondria

Since information on *U. maydis* mitochondria is scarce and fragmented, our first goal was to look for the presence of the classic components of the respiratory chain. A functional approach was used, based on the stimulation of respiration by specific substrates and inhibition of oxygen consumption by known inhibitors of complex I, III or IV. However, the initial experiments with isolated mitochondria gave conflicting results, essentially because we found some methodological problems with the isolation of intact and good quality mitochondria. Hence, we decided to work with digitonin-permeabilised cells. As shown in Table 1, addition of CCCP to digitonin-permeabilised cells did not affect their respiratory activity, suggesting the presence of uncoupled mitochondria (H^+ freely flows across the inner membrane). Nevertheless, the mitochondrial inner membrane was impermeable to small molecules like NADH. Table 1 also shows that *U. maydis* mitochondria contain the four classical respiratory complexes. Oxygen consumption was stimulated by pyruvate-malate (complex I), succinate (complex II), and TMPD-ascorbate (complex IV). As expected, rotenone (complex I), antimycin (complex III) and cyanide (complex IV) inhibited the consumption of oxygen. Besides these enzymes, *U. maydis* also contains an active mitochondrial glycerol 3-phosphate dehydrogenase (Table 1). It is worth mentioning that summation of individual respiratory rates obtained with succinate, pyruvate+malate, or exogenous NADH (130 ng AO min^{-1} AU_{600}^{-1}) is greater than the respiratory rate observed when these substrates are together (Table 1).

3.2. The alternative oxidase in U. maydis

Since the presence of AOX and alternative NADH dehydrogenases in mitochondria is a frequent metabolic strategy used by plants and several fungi, it was not surprising to find out that in intact *U. maydis* cells and in cells permeabilised by digitonin in the presence of AMP and DTT, a significant percentage of mitochondrial respiratory activity was insensitive to rotenone, cyanide or antimycin A,

Table 1
Oxygen consumption by intact and digitonin-permeabilised *U. maydis* cells

Substrate	Respiratory activity (ng AO/min/AU_{600})	N
Intact cells	86±9	7
+KCN (1 mM)	18±8	7
+Antimycin (10 μM)	22±11	3
+Rotenone (15 μM)	40±5	3
Digitonin-permeabilised cells (−AMP −DTT)		
NADH (0.1 mM)	42±7	7
+Ca^{2+} (1 mM)	34±4	4
+ADP (50 nmol)	45±5	5
+CCCP (5 μM)	38±3	4
+Rotenone (3 μM)	44±8	6
+Antimycin (4 μM)	1±1	3
NADPH (0.5 mM)	3±1	3
Succinate (5 mM)	50±9	6
+Antimycin (4 μM)	2±1	5
Pyruvate (10 mM)+malate (10 mM)	38±5	7
+Rotenone (3 μM)	3±1	5
Glutamate (10 mM)+malate (10 mM)	40±10	3
Glycerol 3-phosphate (5 mM)	31±6	3
TMPD (1 mM)+ascorbate (5 mM)	160±12	4
+KCN (1 mM)	2±4	3
+Cytochome *c* (2 μM)	161±6	3
NADH (0.1 mM)+succinate (5 mM)+ pyruvate (10 mM)+malate (10 mM)	76±12	4
Digitonin-permeabilised cells (+AMP +DTT)		
NADH (0.1 mM)	42±4	6
+Antimycin (4 μM)	16±3	3
+KCN (1 mM)	18±4	5

Cells were permeabilised in the presence or absence of AMP (5 mM) and DTT (1 mM).
The permeabilisation procedure is described under Materials and methods.
Respiratory rates represent steady state oxygen consumption. In the case of permeabilised cells, activities are reported after subtraction of the basal respiration (without mitochondrial substrate). Inhibitors were added after the steady state was reached.

pointing to the presence of additional components in the respiratory chain (Table 1). Therefore, we looked at the presence of the AOX by immunoblot (inset in Fig. 1). A single 32-kDa band was evident on the gel, with a molecular mass similar to other plant and fungal AOX, suggesting the presence of this protein in *U. maydis* mitochondria. Interestingly, the AOX activity was lost when *U. maydis* cells were permeabilised in the absence of AMP and DTT (Table 1).

Our next goal was to determine the participation of the AOX in respiration. Oxygen consumption measurements in intact cells are shown in Fig. 1. Respiratory activity of non-permeabilised *U. maydis* cells was 75–80% sensitive to cyanide (1 mM) or antimycin A (10 μM). The 20–25% residual respiratory activity was sensitive to SHAM (250 μM). Since SHAM is a good inhibitor of AOX [24], this result points to the presence of an active AOX in *U. maydis* mitochondria, and shows that mitochondrial respiration is the result of the activity of the classic pathway of quinol oxidation (cytochromes bc_1 and aa_3) and the AOX.
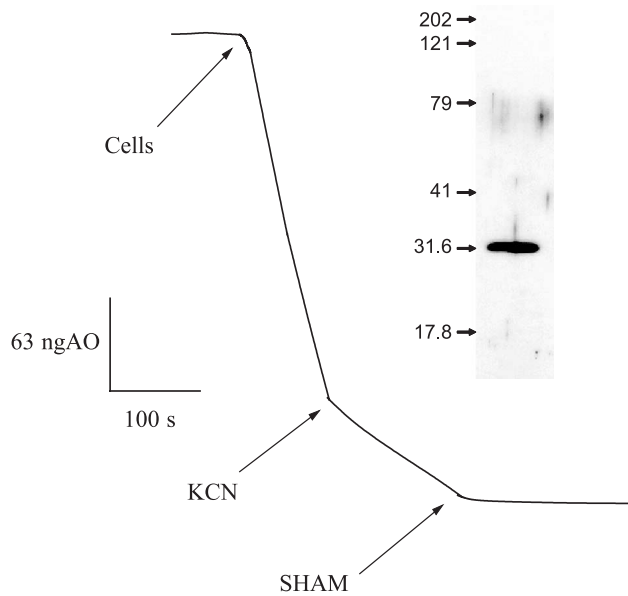
Fig. 1. Inhibition of respiratory activity in intact *U. maydis* cells by cyanide and SHAM. The arrows show the addition of inhibitor. Inset: Immunoblot of *U. maydis* mitochondrial AOX. Electrophoresis was carried out with 50 μg of mitochondrial protein, under denaturing and reducing conditions in 10% polyacrylamide gel.

Fig. 2. Increase in AOX activity after growing *U. maydis* cells in the presence of classic pathway inhibitors. Cells were grown for 24 h in YPD at 30 °C. At this time, cyanide (25 μM) or antimycin (10 μM) was added and incubation at 30 °C continued for further 24 h. Cells were harvested and washed, and their sensitivity to inhibitor evaluated. Black, respiratory activity sensitive to cyanide (1 mM); white, respiratory activity insensitive to cyanide, but sensitive to SHAM (250 μM); gray, oxygen consumption insensitive to both inhibitors. Results from five independent experiments are expressed as the mean±standard error.

Next, we concentrated on the regulation of AOX activity. It is known that many plants and microorganisms increase the expression of alternative components when they grow in the presence of oxygen free radicals [25,26] or inhibitors of the classic respiratory pathway [25,27]. In a similar way, when *U. maydis* cells were grown in the presence of cyanide (25 μM) or antimycin (10 μM), the extent of inhibition by cyanide and SHAM was modified (Fig. 2). Cyanide decreased the oxygen consumption only 5–10%, while inhibition by SHAM increased to 75%. An interesting result is that an important proportion of respiration (~15%) was not associated with mitochondrial terminal oxidase activity, since it was not inhibited by cyanide or SHAM. Since respiratory inhibitors increase the production of oxygen free radicals by mitochondria [28], it is likely that the residual oxygen consumption is the result of the activity of antioxidant enzymes related to oxygen radical handling.

AOX in plants is regulated by α-keto acids, such as pyruvate, and by the redox state of mitochondrial matrix, probably mediated by glutathione or ferredoxin [4,19,20]. In contrast, fungal alternative oxidase is regulated by purine nucleotides, but not by α-keto acids [4,21]. To study the effect of these ligands on *U. maydis* AOX, cells were permeabilised with digitonin in the absence or presence of activators of plant AOX (pyruvate and DTT) and fungal AOX (AMP). When the three ligands were present during cell permeabilisation, the activity of AOX was evident (Fig. 3). In contrast, in the absence of activators, AOX activity was lost (Table 1). Interestingly, the total respiratory rate was similar with or without an active AOX (Table 1). To further discriminate among the three putative activators, both permeabilisation of cells and measurements of *U. maydis*

AOX activity were carried out in the presence of either pyruvate, AMP or DTT (Fig. 3). In agreement with the sensitivity of fungal alternative oxidase to activators, *U. maydis* AOX activity was lost in the presence of pyruvate but it was retained when cells were permeabilised in the presence of AMP. However, it was surprising to find out that DTT protected the enzyme, even though the only putative AOX
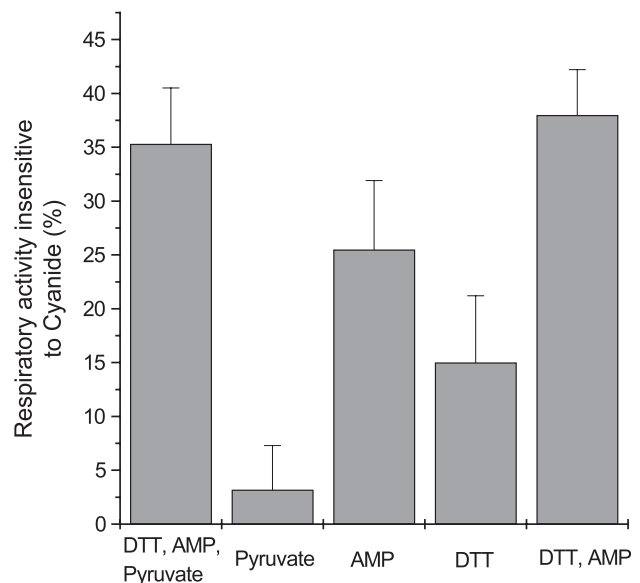
Fig. 3. Effect of AMP, pyruvate and DTT during cell permeabilisation on *U. maydis* AOX activity. Cells were incubated for 1–3 min with digitonin (20 mg/ml), either in the presence or absence of the following activators: pyruvate, 5 mM; AMP, 5 mM; DTT, 1 mM. The respiratory substrate was NADH (0.1 mM).

gene in *U. maydis* genome lacks the regulatory cysteine residues of plant AOX (http://www-genome.wi.mit.edu).

3.3. The alternative NADH dehydrogenase

As mentioned, early studies suggested the presence of an alternative NADH dehydrogenase associated with the respiratory system in *U. maydis* mitochondria, but their topology was an open subject. To address this question, we developed a protocol which takes advantage of the specificity of some inhibitors of NDH-2 and complex I, and the impermeability of inner mitochondrial membrane to small molecules. The results of these experiments are shown in Fig. 4. For permeabilised cells, exogenous NADH increases the rate of oxygen consumption, and this activity was inhibited by flavone, but not by rotenone (Fig. 4A and B). When mitochondrial substrates, such as pyruvate plus malate (10 mM each), were added to the cellular suspension, an increase in respiratory activity was evident. This respiration, in contrast to the one induced by exogenous

Fig. 5. Native blue gel electrophoresis of *U. maydis* mitochondria. Before (A) and after (B) incubation with NADH and MTT. To detect the presence of NADH dehydrogenase activities, the gel was incubated for 30–45 min in Tris–HCl 20 mM, pH 7.4, with 50 μM NADH and 50 μM MTT. Lanes correspond, from left to right, to bovine heart, *U. maydis* and *S. cerevisiae* mitochondria. Molecular weight standards are derived from respiratory complexes of bovine heart mitochondria; our electrophoretic pattern and the pattern shown by Schägger and von Jagow [23] are in close agreement. Complex I, 890 kDa; complex II, 130 kDa; complex III, 500 kDa and complex V, 600 kDa.
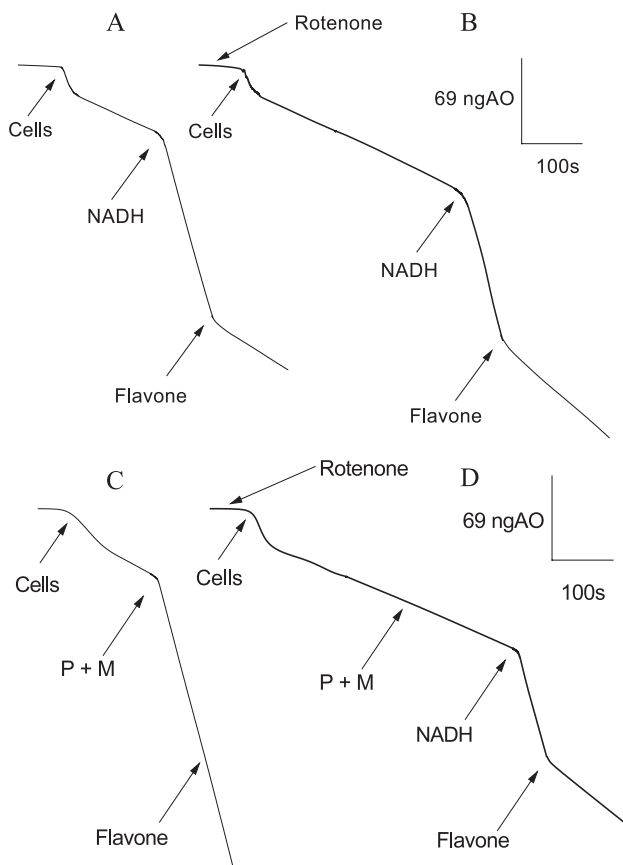

Fig. 4. Effect of rotenone and flavone on internal and exogenous NADH consumption by permeabilised *U. maydis* cells. Respiratory activity stimulated by exogenous NADH (0.1 mM) in the absence (A) or presence (B) of rotenone. Respiratory activity stimulated by pyruvate (10 mM) plus malate (10 mM) in the absence (C) or presence (D) of rotenone. Additions are indicated by arrows. Flavone (250 μM); P+M, pyruvate plus malate (10 mM each); rotenone (3 μM). Cells were permeabilised by digitonin in the absence of AMP, DTT or pyruvate. The cell density used was 3 UA_{600}.

NADH, was inhibited by rotenone but not by flavone (Fig. 4C and D). These results indicate that *U. maydis* mitochondria have two types of NADH dehydrogenases: the alternative NDH-2, facing the cytosolic side of the inner mitochondrial membrane and sensitive to flavone; and the internal rotenone-sensitive NADH dehydrogenase or complex I. This result also indicates that digitonin permeabilised the plasma membrane, leaving intact or disturbing in less proportion other intracellular compartments, such as mitochondria. In contrast to the plant alternative NADH dehydrogenase [30], the respiratory activity with exogenous NADH was not stimulated by Ca^{2+} (Table 1).

Native blue activity gel electrophoresis was important to reinforce the conclusion on the presence of two mitochondrial NADH dehydrogenases. To compare the activity pattern, two controls were included: bovine heart mitochondria, with only complex I present, and *S. cerevisiae* mitochondria, with two external and one internal NDH-2, but no complex I [4,14]. As shown in Fig. 5, bovine heart mitochondria display a single high molecular mass activity band, corresponding with the electrophoretic mobility of complex I [23]. In *S. cerevisiae* mitochondria two activity bands were detected, one of high and the other of low molecular mass. Since *S. cerevisiae* lacks complex I, our data suggest that the NDH-2s in this organism associate to produce supramolecular complexes, a result that has been reported previously [31]. The low molecular mass activity band may represent the minimal activity unit of NDH-2.

In *U. maydis* mitochondria three activity bands appeared. Similar to *Neurospora crassa* complex I, one band showed a molecular mass around 650 kDa. The other band of high molecular mass (580 kDa) probably represents a supramolecular complex of the external NDH-2(s), while the low molecular mass band (approximately 84 kDa) most likely

corresponds to the minimal activity unit of NDH-2, presumably a monomer.

4. Discussion

Very few studies on the energetics or intermediary metabolism have been done with basidiomycetous fungi, even though these organisms are important because they cause several plant diseases. Our study was directed to elucidate the components of the respiratory chain of *U. maydis*, a model fungal phytopathogen responsible of corn smut. The chain is composed of the four classic components, complexes I–IV (evidenced by specific substrate consumption and inhibitor sensitivity), a glycerol 3-phosphate dehydrogenase, and two alternative elements: AOX and the external isoform of NDH-2.

AOX accounts for the 20% residual respiratory activity in *U. maydis* cells after inhibition of the classic pathway of quinol oxidation by antimycin A or cyanide. However, this value can change as a function of the environmental conditions. For example, a characteristic feature of organisms possessing alternative oxidase is that when they grow in the presence of inhibitors of the classic pathway, the expression of the alternative components is increased [25,27]. A similar pattern was observed in *U. maydis*; cells grown in the presence of cyanide or antimycin A showed a four- to fivefold increase in the activity of AOX, while the activity of the classic pathway was decreased five- to sixfold. Since respiration in these cells is partially uncoupled to the synthesis of ATP, it opens some basic questions on the biology of *U. maydis*.

Plant AOX is regulated by α-keto acids (such as pyruvate) and the mitochondrial redox state. The modulation by α-keto acids is the result of the covalent interaction of pyruvate (in the form of thiohemiacetal) with a highly conserved cysteine residue [10,19,20]. The reduction of this cysteine residue by DTT or β-mercaptoethanol results in the activation of AOX. In contrast, fungal AOX does not have this important cysteine residue [4,21], in agreement with the lack of regulation of the fungal enzyme by pyruvate and reducing agents on this enzyme. As expected, the results showed that *U. maydis* enzyme followed the fungal behavior: its activity was stimulated by AMP and pyruvate had no effect during cell permeabilisation. A surprising result was the preservation of AOX activity by DTT. This effect was specific for DTT, since β-mercaptoethanol did not protect the enzyme during cell permeabilisation. It is likely that activation of plant AOX and protection of *U. maydis* AOX activity by DTT follow different mechanisms, since the single putative AOX gene in *U. maydis* lacks the regulatory cysteine residues found in plant AOX.

It has been shown that the responses of classic and alternative quinol oxidizing pathways are interdependent, in such a way that inhibition of one pathway results in activation of the other [29,32,33]. In *U. maydis* mitochon-

dria we found the same phenomenon. Respiration of permeabilised cells with or without an active AOX was nearly the same, suggesting that in the presence of an active AOX the activity of the cytochrome pathway decreases to some extent, resulting in no change in total oxygen consumption. Therefore, when there is no AOX activity, quinol is oxidized by the classic pathway, but when AOX becomes active, both pathways compete for quinol, bringing about a decrease in the activity of the classic pathway.

Two mitochondrial NADH dehydrogenase activities were detected in *U. maydis* mitochondria: (a) one sensitive to flavone, facing the cytosolic side of the inner mitochondrial membrane, showing the presence of at least one external alternative NADH dehydrogenase, and (b) an internal, rotenone-sensitive dehydrogenase, indicating the presence of complex I. As with the classic and alternative quinol oxidizing pathways, the quinone reducing pathways (each one composed by a dehydrogenase) do not present an additive behavior. The coexistence of several types of NADH dehydrogenases is a common strategy of microorganisms and plants. In *S. cerevisiae* mitochondria one internal and two external alternative dehydrogenases are involved in NADH-dependent respiration, but complex I is absent [4]. On the other hand, up to two internal and two external alternative dehydrogenases, in addition to complex I [10,34], are responsible for the respiratory activity of plant mitochondria. Interestingly, *Yarrowia lipolytica* mitochondria [35] have the same distribution of dehydrogenases as *U. maydis* mitochondria. The biological value of these apparently redundant mechanisms of NADH-quinone electron transfer is not fully understood.

The sequences of some NADH dehydrogenases show a Ca^{2+} binding EF hand motif [5]. In fact, the alternative NADH dehydrogenase of plants is activated by Ca^{2+} and, depending on the isoform, calcium requirements are different among them [30]. The external enzyme in *N. crassa* mitochondria contains a putative Ca^{2+} binding motif, and it is presumed to be Ca^{2+}-sensitive [36]. However, some of these results must be taken with care, since activation by calcium was observed on mitochondrial functions, but not on purified enzyme. Exogenous NADH-dependent respiratory activity in *U. maydis* permeabilised cells is not sensitive to Ca^{2+}, implying two possibilities: (1) NDH-2 is not activated by Ca^{2+}, or (2) NDH-2 is activated by Ca^{2+}, but has a very low control flux coefficient, which means that even when its activity can be decreased or increased two or three times, there is not a parallel change in respiratory flux.

The special topology of the external alternative NADH dehydrogenase has been taken as an indication of the role of this enzyme in cellular metabolism. Mechanisms of transport of redox equivalents from cytosol to mitochondria are different in yeast and mammalian cells. Aspartate-malate shuttle is absent in fungi (those studies were made in ascomycetous fungi). However, several new shuttles arose in yeast cells to account for the lack of aspartate-malate shuttle, like the acetaldehyde-ethanol, malate-oxaloacetate, and the

external alternative NADH dehydrogenase. The relative importance of these shuttles depends on the metabolic state of the cell, and probably varies from one organism to another. Studies made in S. cerevisiae by Bakker et al. [14] and Luttik et al. [15] indicate the important role of the external alternative NADH dehydrogenase in the physiology of this yeast. Our experiments revealed that in addition to the external NADH dehydrogenase, U. maydis contains the glycerol 3-phosphate dehydrogenase shuttle for transport of redox equivalents into mitochondria.

A recent breakthrough in the field of basidiomycete fungi is the sequencing and release of the U. maydis genome by The Whitehead Institute in collaboration with Bayer [37]. With this information at hand, a BLAST analysis was performed on the genome sequence of U. maydis, using the amino acid sequence of the S. cerevisiae internal NADH dehydrogenase (NDI1) [38]. Three genes coding for proteins sharing high identity (33–49%) and low E values (2×10^{-94}–1×10^{-48}) were identified. In addition, it is predicted that these proteins contain a mitochondrial target sequence [39] and the two putative adenine nucleotide binding motifs characteristic of this protein family. After removal of the mitochondrial target sequence, the molecular masses of the mature proteins were calculated as 62.7, 69.4, and 58 kDa. Since these values are approximately the sizes of the molecular weights obtained by native blue electrophoresis, this result suggests that the minimal active unit of the NADH dehydrogenase is the monomer. Work is in progress to establish which one of the putative NADH dehydrogenase genes is expressed in U. maydis yeast cells growing in YPD medium.

Acknowledgements

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3.2 El papel metabólico de la AOX en *Ustilago maydis*

The physiologic role of alternative oxidase in *Ustilago maydis*

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Alternative oxidase (AOX) is a ubiquitous respiratory enzyme found in plants, fungi, protists and some bacterial species. One of the major questions about this enzyme is related to its metabolic role(s) in cellular physiology, due to its capacity to bypass the proton-pumping cytochrome pathway, and as a consequence have great energy-wasting potential. In this study, the physiological role and regulatory mechanisms of AOX in the fungal phytopathogen *Ustilag maydis* were studied. We found evidence for at least two metabolic functions for AOX in this organism, as a major part of the oxidative stress-handling machinery, a well-described issue, and as part of the mechanisms that increase the metabolic plasticity of the cell, a role that might be valuable for organisms exposed to variations in temperature, nutrient source and availability, and biotic or abiotic factors that limit the activity of the cytochrome pathway. Experiments under different culture conditions of ecological significance for this organism revealed that AOX activity is modified by the growth stage of the culture, amino acid availability and growth temperature. However, nucleotide content, stimulation of AOX by AMP and respiratory rates obtained after inhibition of the cytochrome pathway showed that fungal/protist AOX is activated under low-energy conditions, in contrast to plant AOX, which is activated under high-energy conditions. An estimation of the contribution of AOX to cell respiration was performed by comparing the steady-state concentration of adenine nucleotides, the mitochondrial membrane potential, and the respiratory rate.

Ustilago maydis is a fungal phytopathogen that infects wild and commercial types of corn (*Zea teocintle* and *Zea mays*, respectively), and is related to other fungal pathogens of great economic significance. As a pest, a culinary delicacy or a model organism it is considered an interesting subject, and a large amount of information on diverse aspects of its biology is available [1–3]. Despite this, many issues are not understood, in particular its energy and intermediary metabolism.

We have previously described the organization of the *U. maydis* mitochondrial respiratory chain [4]. Among our major results were the identification of alternative oxidase (AOX) and the external isoform of alternative NADH dehydrogenase in this organism. These enzymes are widespread among eukaryotes [5,6] and can be found in some bacterial species [7,8], suggesting a significant metabolic relevance. However, their participation in metabolism has been difficult to

understand because they are not directly associated with ATP synthesis. The experimental evidence shown in this study illustrates that the main metabolic roles of AOX in *U. maydis* are: (a) the prevention of reactive oxygen species (ROS) production; and (b) to increase the metabolic plasticity of the cell, avoiding metabolic collapse under conditions in which the cytochrome pathway is impaired. In addition, some culture conditions were used to understand the major regulatory aspects of AOX and the impact of AOX on cell economy. A method to evaluate the participation of AOX in whole-cell respiration is described.

Results

Despite the extensive characterization of plant and fungal AOX, its contribution to respiration has been difficult to determine. Jarmuszkiewicz *et al.* [9] described a quantitative method to discriminate between the activities of the cytochrome pathway and AOX, based on selective activation of the cytochrome pathway by ADP. However, this method can be applied only to isolated mitochondria under conditions where complex I is not active. In a different approach, the oxygen discrimination method was used to determine the quantitative contribution of AOX to respiration in mitochondria and intact cells [10–12]. The results found were in agreement with previous theory and with experimental data, and gave valuable information. However, this method has some problems: it is assumed that $^{18}O/^{16}O$ discrimination by the cytochrome pathway and AOX is constant under every condition tested, i.e. along the range of quinone pool redox states [9], with all the respiratory substrates (NADH or succinate), or at different activation states of AOX. Because a practical method of determining the contribution of each pathway to respiration in intact cells or tissues is not available, we measured the capacity of the two respiratory systems, considered as the uncoupled respiratory activity in the presence of KCN (AOX capacity) or *n*-octilgallate (nOg) (cytochrome capacity). The uncoupler is required to evaluate respiratory activity without interference from the proton gradient generated by the enzymes located upstream of the ubiquinone pool, as complex I, but as mentioned in the Experimental procedures, addition of uncoupler did not affect AOX capacity, suggesting that other nonproton-pumping ubiquinone reductases, such as succinate dehydrogenase or alternative NADH dehydrogenase, make a large contribution to respiration. However, it is important to stress that complex I is the only internal NADH dehydrogenase in this fungus [4], so many processes depend on this enzyme,

including the Krebs cycle and fatty acid and amino acid metabolism. Furthermore, as shown here, its sole activity is capable of sustaining mitochondrial ATP synthesis.

However, the reported rates cannot be conclusively considered maximal, because they depend on many other processes such as ubiquinone reduction pathways, the tricarboxylic acid cycle glycolytic activity, metabolite transport and the endogenous substrate concentration.

Factors that modulate AOX activity

Like many other free-living organisms, *U. maydis*, a cosmopolitan organism, is subjected to changes in several environmental parameters, for example, substrate source and availability, temperature, and the cell population density of its own and other species. The aim of the first part of this study was to understand how these parameters modify the respiratory activities of the cell, and how these changes can be interpreted as metabolic adaptations.

Temperature

Large changes in temperature can be found in the ground, from $< 0\ °C$ at night to $> 40\ °C$ at noon. It was therefore of interest to evaluate the mitochondrial respiratory activity in cells grown at temperatures ranging from 28 to 34 °C. As shown in Fig. 1A, the cytochrome pathway capacity (165–190 nmol $O \cdot min^{-1} \cdot A_{600}^{-1}$) and the uninhibited cell respiratory rate (140–160 nmol $O \cdot min^{-1}$) were not affected by growth temperature. In contrast, AOX capacity showed a great dependence on this parameter, displaying sigmoid behavior and reaching the uninhibited respiration rate in the cells grown at 34 °C.

The increase in AOX capacity produced a large change in the response of cellular respiration to inhibitors (Fig. 1B). In cells grown at 28 °C, cell respiratory rate was inhibited 80% by cyanide, and almost all the residual activity was sensitive to nOg (or salicylhydroxamic acid), as reported previously [4]. If the order of the additions is reversed, there is only a slight response to nOg (= 7%), and subsequent addition of cyanide inhibited respiration. For cells cultured at 34 °C, addition of only one inhibitor did not affect the respiratory rate, and two must be present to inhibit cell respiration, indicating that the AOX and cytochrome pathways can fully compensate for each other.

The data suggest that the activity of the ubiquinone reduction pathways could be greater than the capacities of the AOX or cytochrome pathways, and it is
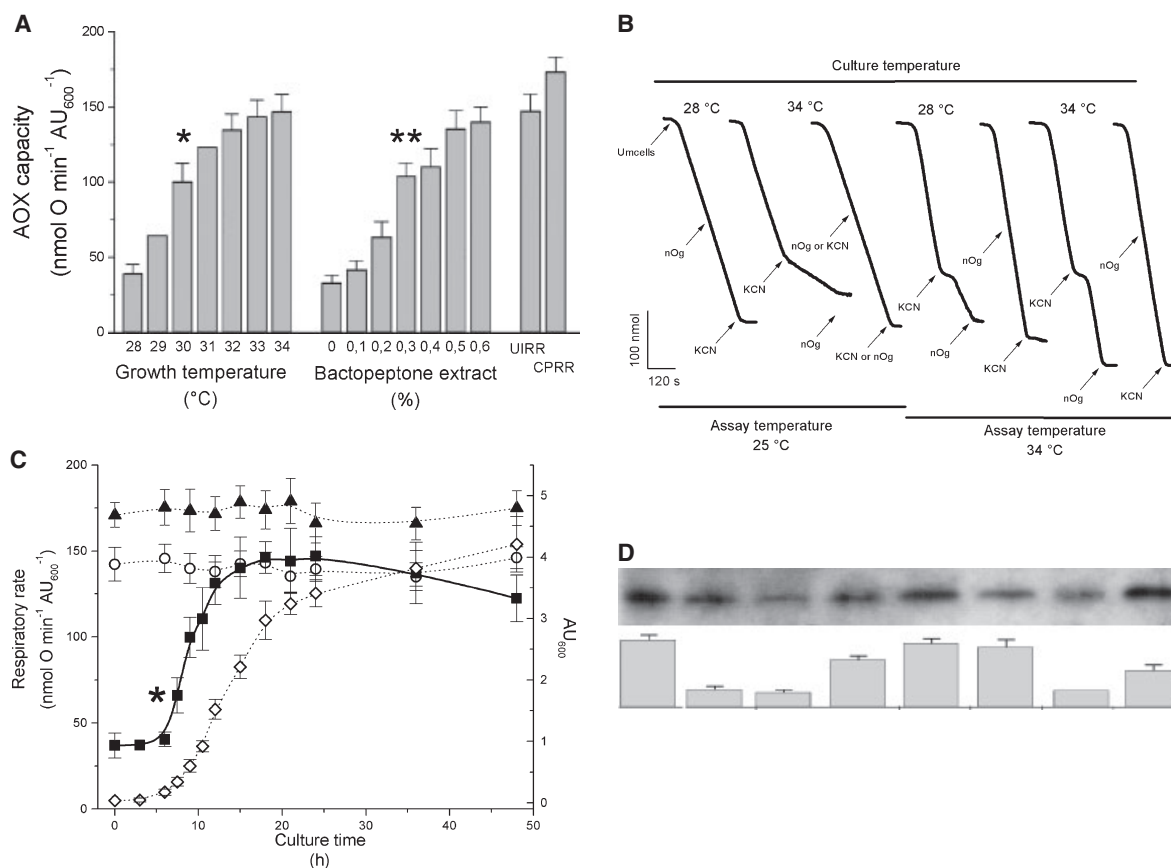
Fig. 1. Ecological factors that modulate AOX activity in *U. maydis* cells. (A) Effect of growth temperature and medium richness on AOX activity in *U. maydis*. The capacities of the AOX and cytochrome pathways were obtained at 34 °C as described in Experimental procedures, in the presence of the uncoupler PCP and with either nOg or KCN, in cells grown for 24 h (stationary phase of growth) at the indicated temperatures or bactopeptone concentrations. CPRR, cytochrome pathway respiratory rate; UIRR, uninhibited cell respiratory rate. * Significant difference in AOX capacity compared with cells cultured at 28 °C ($P < 0.001$). ** Significant difference in AOX capacity compared with cells cultured in the absence of bactopeptone ($P < 0.001$). (B) Respiratory traces of *U. maydis* cells grown for 24 h, at 28 or 34 °C, showing the sensitivity to inhibition by cyanide (1 mM) and nOg (2 μM), and the effect of the assay temperature. Oxymetric experiments were performed at 25 or at 34 °C in KME medium. (C) Phase growth dependence of the respiratory activities. Cells were grown in YPD medium at 34 °C, and at the indicated times were harvested and the capacities of the AOX and cytochrome pathways were analyzed. * Significant difference in AOX activity at the indicated culture time compared with initial AOX capacity ($P < 0.001$). ○, whole-cell respiration; ▲, cytochrome pathway capacity; ■, AOX capacity; ◇, cell density. Oxymetric measurements were carried out at 34 °C. (D) Expression levels of AOX in *U. maydis* mitochondria. Isolation of mitochondria is described in Experimental procedures. Lanes 1 and 2 correspond to the mitochondrial preparation from cells cultured in YPD medium for 24 h, at 34 and 28 °C, respectively. Lane 3 corresponds to mitochondria obtained from cells grown at 34 °C in minimal medium, and in lanes 4 and 5 the minimal media was supplemented with 0.3 and 0.6% of bactopeptone, respectively. Lane 6 corresponds to the mitochondrial preparation from cells cultured in YPD medium at 28 °C that were and exposed to 10 μM of H_2O_2 for 40 min. In lanes 7 and 8 the expression levels of AOX were investigated in mitochondria obtained from cell grown in YPD medium at 34 °C for 3 and 8 h, respectively. The lower part of the figure shows the normalized AOX capacity of each sample.

possible that ubiquinol oxidation has a high control on the flux, because the respiratory rate seems to be independent of the nature, and even of the number, of ubiquinol-oxidizing pathways, i.e. in cells with high AOX activity, oxygen consumption is nearly the same in the absence of inhibitors (with the two terminal oxidases), in the presence of cyanide (with AOX as terminal oxidase), or in the presence of nOg (with the cytochrome pathway as the terminal oxidase).

To gain further insight about the respiratory activities during cell culturing, oxymetric measurements were performed at 28 and 34 °C. The increase in assay temperature from 25 to 28 °C produced a 1.7–1.9 × increase in the uninhibited cell respiratory rate and 3.5–4.3 × increase in AOX capacity. A further increase in assay temperature (to 34 °C) resulted in a small increase (5–11%) in the rates obtained at 28 °C. One feature produced by the increase in assay temperature

was a transitory inhibition of respiration after the addition of cyanide, which was not apparent with nOg (Fig. 1B).

Growth rate

In cells grown at 28 °C, the uninhibited respiratory activity, cytochrome pathway capacity and AOX capacity ($\approx$ 35 nmol O·min^{-1}·A_{600}^{-1}) showed a constant rate along the growth curve (data not shown). In contrast, a sharp increase in AOX activity, at the beginning of the exponential growth phase, was seen in cells cultured at 34 °C, reaching a maximum value at $\approx$ 24 h, and decreasing slowly afterwards (Fig. 1C). As in the other cases, the uninhibited respiratory activity and the cytochrome pathway capacity were constant.

Growth medium

The effect of the carbon and nitrogen source on AOX activity was studied in cells cultured at 34 °C in minimal media, supplemented with different concentrations of glucose (0.1–5%) or yeast extract (0–5%). AOX activity was kept low and constant under these conditions (not shown). However, the addition of bactopeptone (0–0.6%) led to a large increase in AOX capacity (Fig. 1A) and, as in the other cases, the uninhibited respiratory activity and cytochrome pathway capacity were unaffected by this condition.

AOX protein level control

As can seen in Fig. 1D, one of the major control mechanisms of AOX activity seems to be at the protein level, because in many cases, a good correlation was found between AOX activity and the signal obtained in the immunoblot. For example, mitochondrial preparations from cells grown for 24 h at 28 °C in YPD medium (lane 2) and cells cultured for 3 h at 34 °C (lane 7) showed a smaller amount of AOX protein and a lower AOX capacity than mitochondria from cells grown for 24 h in YPD medium at 34 °C (lane 1). In the same way, an increase in AOX was found in cells grown in the presence of higher concentrations of bactopeptone extract (lanes 3–5).

However, in other cases, the amount of AOX protein did not correlate with AOX activity, for example, cells incubated for 40 min in the presence of 10 μM H_2O_2 (lane 6), showed a 5–6 $\times$ increase in AOX activity (Fig. 3D), but the protein band did not display any significant variation, suggesting that the amount of AOX was regulated at the activity level. Also, cells cultured

for 8 h at 34 °C showed a large amount of AOX, but its capacity was reduced (lanes 8), pointing to an inactive pool of AOX in this condition that is activated as the cell reaches other growth stages.

AOX and ROS homeostasis

It has been extensively documented that AOX makes a large contribution to the prevention of ROS production [13–17]. To study the potential role of AOX as an antioxidant enzyme in *U. maydis*, experiments were performed to evaluate the effect of AOX on both the production of a single ROS (H_2O_2) and the activities of some ROS-handling enzymes, for example, catalase and glutathione reductase, using cells with a considerable range of AOX capacities.

Production of H_2O_2 in the presence of mitochondrial inhibitors agrees with the role of AOX in the prevention of oxidative stress. Indeed, ROS production was increased after the addition of KCN, but was lower in cells showing high AOX capacity (Fig. 2A), supporting the idea that electrons can be redirected to AOX. Furthermore, H_2O_2 production in the presence of nOg was greater in cells with high AOX capacities (Fig. 2A), and a comparison between H_2O_2 production in the presence of nOg ($H_{2}O_{2\ nOg}$) and in the presence of cyanide ($H_{2}O_{2\ KCN}$) revealed that inhibition of AOX led to a greater increase in ROS production than inhibition of the cytochrome pathway (Fig. 2B). As expected, the highest ROS production was found when the two inhibitors were added together.

Despite the antioxidant activity of AOX, a correlation between basal H_2O_2 production (in the absence of any inhibitor) and its activity was not found, raising two possibilities: (a) our method was not able to detect small differences in ROS production that might be important for cell metabolic homeostasis, or (b) cell metabolism maintains ROS levels within limits that can be tolerated by cells. However, the results shown in Fig. 3C strongly suggest that AOX is able to reduce basal ROS production, because a negative relationship between the activities of catalase and glutathione reductase and AOX capacity was found. The increase in AOX capacity seen following treatment with H_2O_2 (Fig. 3D) provides further support for this interpretation.

AOX and the energy status of the cell

To understand the impact of AOX on the energy status of the cell, the effect of several metabolic inhibitors or uncouplers [KCN, nOg, pentachlorophenol (PCP) and iodoacetate (IAA)] on the adenine nucleotide con-
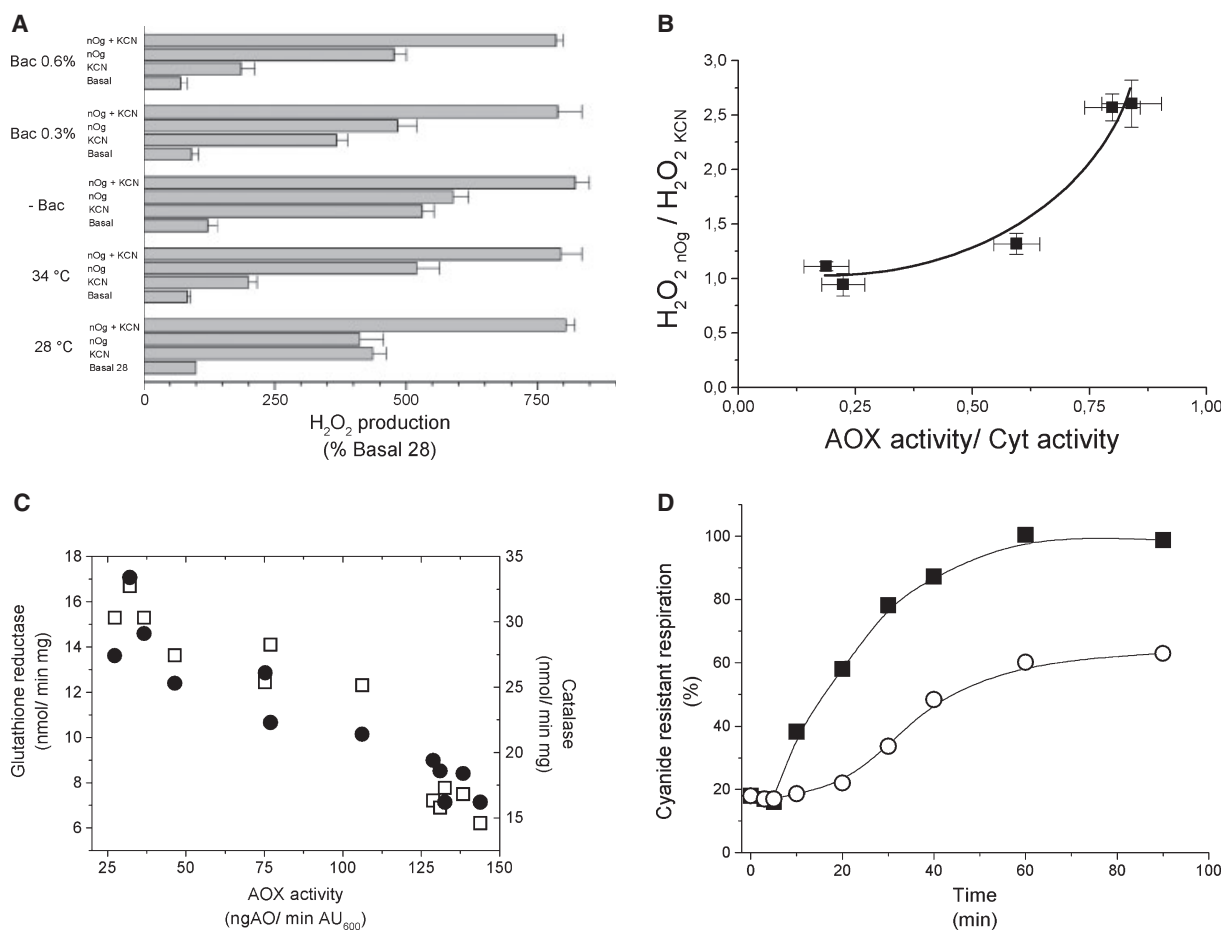
Fig. 2. ROS production by *U. maydis* cells. (A) Fluorometric determination of H_2O_2 production in the absence of inhibitors (basal) or in the presence of nOg (2 μM) and∕or KCN (1 mM), in cells cultured in YPD medium at 28 or 34 °C, or in minimal medium supplemented in some cases with bactopetone (0.3 or 0.6%) at 34 °C, that showed significant differences in AOX activity. The intrinsic fluorescence of the nonloaded cells was subtracted in all the cases, and the fluorometric signal was normalized with respect to the basal production of H_2O_2 by cells grown in YPD medium at 28 °C (Basal 28). (B) Comparison of the production of H_2O_2 via the AOX and cytochrome pathways. The production of H_2O_2 by cells showing different AOX/cytochrome pathway capacity ratios was determined in the presence of nOg ($H_2O_{2\ nOg}$) or with KCN ($H_2O_{2\ KCN}$) to evaluate the contribution of the cytochrome pathway or the AOX to the ROS production. (C) Activity correlation between ROS-handling enzymes with AOX. The activity of catalase and glutathione reductase were determined as described in Experimental procedures, following either the decrease in NADPH or the production of O_2 in cells with different AOX capacities. (D) H_2O_2 induction of AOX activity. Cells were cultured in YPD medium at 28 °C. After harvesting cells were incubated in KME medium in the presence or absence of H_2O_2 (○, 1 μM; ■, 10 μM). At the indicated times, an aliquot was withdrawn and the AOX capacity was determined. Because KCN inhibition is more pronounced at lower temperatures, oxymetric measurements were taken at 25 °C.

tent of cells displaying different AOX activities was measured. A complete description of the results is shown in Fig. S1. The major findings are: (a) total ATP concentration was similar in cells cultured under diverse conditions, independent of AOX activity (Fig. 2A, and supplementary Fig. S1); (b) inhibition of the cytochrome pathway by KCN led to a decrease in ATP concentration, but this change was smaller in cells with a high AOX capacity (Fig. 2A); (c) inhibition of AOX did not lead to a significant change in

ATP concentration (Fig. 2A,B); and (d) inhibition of both the cytochrome pathway and AOX led to a 40–50% decrease in ATP content (Fig. 2A, supplementary Fig. S1).

To further characterize the energy metabolism of this organism, the uncoupler PCP and the glycolytic inhibitor IAA were tested (Fig. 2B). Interestingly, IAA had no appreciable effect on ATP content, either alone or in the presence of nOg, but produced a large effect in the presence of KCN, PCP or nOg∕KCN (Fig. 2B),

A

B

C

Discussion

Detailed study of the metabolic function of AOX requires an examination of the factors that modulate its activity. Indeed, these studies have been used to support some proposals about the role of AOX [18–25]. This study shows the regulation of AOX under conditions that might be of ecological significance for the cosmopolitan phytopathogen *U. maydis*, and comparison of the metabolic properties of cells showing differences in AOX capacity.

Carbon flux and AOX regulation

It is evident that AOX activity must be strictly regulated at the protein and expression levels, because a fully active uncontrolled enzyme would reduce mitochondrial ATP synthesis, and consequently the biomass yield. Regulation of AOX activity should be especially important in cases in which the economy of the cell is compromised. Indeed, a recombinant strain of *Schizosaccharomyces pombe* that overexpresses AOX shows a decrease in growth rate and biomass yield in minimal media [26]. In contrast, recombinant *Trypanosoma brucei* overexpressing AOX shows a wild-type phenotype [27], presumably because this organism has regulatory mechanisms that adjust AOX activity to meet the metabolic requirements of the cell.

In *U. maydis*, the nature and amount of the nitrogen source seem to regulate AOX activity, because cells cultured in the presence of high concentrations of

pointing to an important glycolytic activity that can be increased at conditions under which mitochondrial ATP synthesis is impaired.

To obtain a deeper understanding of mitochondrial function in the intact cell, we studied mitochondrial membrane potential (Fig. 2C). As expected, the uncoupler (PCP) abolished the fluorometric signal sensitive to membrane potential (inset Fig. 2C). However, the addition of KCN produced a transient decrease in membrane potential; nOg had no effect.

bactopeptone showed substantial AOX capacities, possibly because amino acid biosynthesis or nitrogen assimilation are expensive processes for cells cultured in minimal media, but not for cells cultured in rich media, which in turn suggests that AOX may decrease mitochondrial ATP synthesis. However, our results indicate that AOX does not participate in respiration to any great extent (see below), and in order to prevent the energy-wasting potential of AOX the cell either controls expression of this enzyme (as in minimal conditions) or regulates its activity (in a rich medium).

AOX as a major control site of ROS production

It is known that the semiquinone radical, produced in the catalytic cycles of complexes I [28,29] and III [30], can react with oxygen, producing the superoxide anion [31,32]. Indeed, mitochondrial metabolism seems to be a major source of ROS [33,34], and a wide range of conditions have been described under which the lifetime of the semiquinone ion is increased, as is ROS production, for example, during inhibition of the cytochrome pathway or at high redox states of the ubiquinone pool [35]. AOX activation has been described as one mechanism to control ROS production [13–17], because AOX bypasses formation of the semiquinone ion by the cytochrome pathway, decreases the QH_2/Q ratio, and consequently diminishes production of the superoxide anion.

In *U. maydis* cells, AOX also seems to participate in oxidative stress management, because production of H_2O_2 increased greatly following inhibition of AOX (4–6 times), even in cells with a low AOX capacity (Fig. 2A). In addition, AOX activity was correlated with a smaller effect of cyanide on the oxidative rate (due to electron redistribution), this is the reason for the curve Fig. 2B, which shows the great potential of AOX as an antioxidant enzyme. However, AOX seems to contribute little to cell respiration under regular conditions (discussed below), suggesting that mitochondrial ROS production might follow a nonlinear relationship with the redox state of the ubiquinone pool, as suggested previously [31]. Following this interpretation, a small change in the redox state of the pool will produce a large variation in ROS production.

By contrast, the fluorometric method used here to determine H_2O_2 production was not able to detect differences in the basal rate of ROS production in cells with different AOX activities. However, evaluation of the activities of some ROS-handling enzymes (documented to respond, at the expression level, to ROS) [36–38], showed that cells with high AOX activity had lower activities of catalase and glutathione reductase, suggesting that AOX can reduce basal oxidative stress significantly. In agreement with this is the increase in AOX activity at the beginning of the logarithmic growth phase, probably to a decrease in ROS production, which is able to inhibit DNA replication [39].

The impact of AOX on cell bioenergetics and its contribution to respiration

Careful examination of the effect of AOX on the energy status of the cell showed some interesting results (Fig. 3A): (a) AOX inhibition did not change the ATP concentration, (b) ATP content was similar in cells with large differences in AOX capacity, (c) the effect of cytochrome pathway inhibition on ATP concentration was attenuated in cells showing high AOX activity, and (d) inhibition of mitochondrial metabolism (nOg + KCN) resulted in a 50% decrease in ATP concentration.

The lack of an effect of AOX inhibition on both intracellular ATP concentration and cell respiratory rate (Fig. 1B) led us to understand the participation of AOX in respiration in the intact cell. The results agree with the view that, in the absence of inhibitors, electrons flow mainly via the cytochrome pathway, as reported using the oxygen discrimination method in other systems [10–12]. To illustrate our point, we formulated some hypotheses that cover many metabolic circumstances. In the simplest case, if electrons flow mainly via AOX or equally via both pathways, then inhibition of AOX would increase the ATP content (if the respiratory rate is kept constant), because the electrons would be redirected to a proton-pumping/ATP-synthesizing pathway. However, considering that ATP may decrease the activity of complex IV [40] and the phosphorylation system rate, it is possible that, upon AOX inhibition, a large increase in ATP concentration could be seen, followed by a decrease in respiratory rate and adjustment in the ATP concentration, possibly to reach a value close to that observed in the uninhibited cell. However, a complementary scenario can be found in which cells buffer the nucleotide content during AOX inhibition, either by modulating the glycolytic rate (Pasteur effect) or by decreasing the thermodymamic efficiency of oxidative phosphorylation (by proton slip or proton leak), so that changes in the respiratory rate will not influence the ATP content. We discarded these possibilities discarded in our system based on the following facts: (a) inhibition of glycolysis by IAA does not modify ATP content when either the AOX (nOg) or cytochrome pathways (KCN)

are inhibited, but there is a large decrease in ATP concentration when mitochondrial metabolism is inactive (nOg + KCN), suggesting that the glycolytic rate can sustain ATP synthesis to a great extent; (b) in cases in which mitochondria regulate the thermodynamic efficiency of oxidative phosphorylation, a change in the proton electrochemical gradient is seen [41], but we have shown that the addition of nOg did not change the fluorescence of the mitochondrial membrane potential probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (Fig. 2C), which shows that AOX inhibition does not redirect the electron flow, and that the electron flow via the cytochrome pathway is sufficient to maintain a maximal transmembrane potential, even when the AOX pathway is active.

The transient inhibition of cellular respiration following the addition of KCN (Fig. 1B) further supports to the idea that the contribution of AOX to cell respiration is low, because it clearly illustrates the small activity of AOX before the addition of KCN and its activation following inhibition of the cytochrome pathway. Indeed, the low participation of AOX in respiration explains why cell ATP content and AOX capacity act independently (Fig. 2A), because the impact of AOX on cell bioenergetics should be negligible. It is worth mentioning that our study offers a new way of qualitatively measuring the contribution of AOX to cell or tissue respiration, based on widely used and fully characterized techniques that are accessible to many laboratories around the world, namely, oxymetric measurements, enzymatic analysis of the nucleotide content and fluorometric measurements of mitochondrial membrane potential.

AOX as a mechanism for increasing the metabolic plasticity of the cell

It is recognized that the AOX and cytochrome pathway activities do not show additive behavior, and inhibition of one pathway increases the activity of the other (to an extent determined by its kinetic parameters) and the change in respiratory activity is attenuated [4]. Thus, inhibition of any pathway could be compensated for by the complementary route. The increase in AOX capacity, caused by high culture temperature or amino acid supply, produced an increase in the compensatory effect of both pathways. For example, in cells cultured in YPD medium at 34 °C, the inhibition of one pathway can be fully compensated for by the other, because both capacities are similar, and as a consequence respiration is blocked only

if the inhibitors of the two pathways are put together. By contrast, in cells cultured at 28 °C, AOX inhibition can be fully compensated for by the cytochrome pathway, but AOX cannot compensate completely for the inhibition raised by KCN. This compensatory effect increases the metabolic plasticity of the cell, allowing the intermediary metabolism to proceed at conditions under which the cytochrome pathway is impaired. In fact, may biotic interactions may reduce the activity of complex III or complex IV, for example, as the active production of ROS or reactive nitrogen species during plant and animal defense against pathogens [42], cyanide production by many micro-organisms and plants [43,44], and antimycin or methoxyacrilate production by soil bacteria, particularly *Mixobacteria* and the genus *Streptomyces* [45–49]. In addition, many abiotic factors as pH, drought, temperature [18–25] and the availavility of copper [50] have a negative effect on the cytochrome pathway. The decrease in mitochondrial metabolism caused by these factors has serious consequences for many processes, in particular activity of the tricarboxylic acid cycle and the metabolic pathways connected to it, for example lipid and amino acid biosynthesis. In addition, inhibition of the cytochrome pathway should trigger a fermentative metabolism, an expensive process in which the reoxidation of NADH is coupled to the reduction and efflux of an organic molecule from the cell. As shown here, the compensatory effect of AOX supports mitochondrial respiration and ATP synthesis, because cells with a high AOX capacity buffer the ATP content after the addition of KCN, indicating that AOX sustains the activities of complex I, complex V, the tricarboxylic acid cycle and glycolysis. Indeed, mitochondria experienced a transient depolarization after inhibition of the cytochrome pathway, followed by partial recovery within few seconds (Fig. 2C), resembling the inhibition of respiration by KCN. In fact, in *U. maydis*, AOX is able to sustain other complex processes, because these cells are able to grow in the presence of cytochrome pathway inhibitors and chloramphenicol [4].

Our results agree with the overflow mechanism proposed by Bahr & Booner [51], in the sense that one of the roles of AOX is to avoid over-reduction of the ubiquinone pool, becoming active only when the ubiquinol concentration is high [9], but because a high or complete reduction of the ubiquinone pool might be difficult to achieve *in vivo*, its contribution to respiration is low, due to the different mechanisms counteracting large changes in ROS production. However, because electrons can be redirected to both pathways, this proposal should be treated with caution, as pointed out previously [52].

AOX regulation in fungi and plants: metabolic implications

Regulation of plant AOX has been investigated, and detailed characterization of the molecular mechanisms involved in this process is available. The activity of plant AOX is increased considerably upon treatment with reducing agent, owing to the reduction of an intersubunit disulfide bridge, in the inactive dimeric enzyme [53]. In the reduced form the protein is further activated by α-ketoacids, in particular pyruvate, which reacts with a reduced sulfydryl residue, producing a thiohemiacetal [54]. It is possible that formation of the thiohemiacetal is catalyzed by AOX itself, because the stimulatory effect is seen in inside-out mitochondrial particles and in the solubilized enzyme [55]. However, the physiological mechanism involved in the reduction of the intersubunit disulfide bridge of AOX was unclear until recently. It has been shown that AOX can be activated by some tricarboxylic acid cycle intermediaries such as malate and isocitrate, via production of the reducing agent NADPH [56,57], possibly mediated by thioredoxin and gluthathione [53,55]. Indeed, some plants contain a mitochondrial type of thioredoxin (thioredoxin h) that can specifically activate AOX [58]. Experiments developed with the Q electrode indicated that AOX activity was appreciable only at high QH_2/Q ratios [59], and that activation by the reducing treatment and by pyruvate seems to be mixed, increasing both the apparent affinity for ubiquinol and the V_{max} of AOX, making the enzyme more active at low QH_2/Q ratios [60]. By contrast with the plant enzyme, fungal and protist AOX do not form covalent dimers [61] and are not stimulated by pyruvate or by reducing treatment [62]. Instead, they are stimulated by purine nucleotides such as ADP, AMP and GMP [62].

This study shows that regulation of AOX in fungi and plants seems to respond to different metabolic stimuli. In plants, activation appears under high energy conditions [63], for example in the onset of photosynthesis, where oxidative phosphorylation is restricted by the availability of ADP. In this case, the levels of QH_2 and NADH are high, producing an increase in the steady-state concentration of some intermediaries, for example pyruvate, malate or isocitrate, which in turn elevate the steady-state concentration of NADPH, glutathione and thioredoxin. The increase in pyruvate and thioredoxin concentrations should activate AOX, preventing collapse of the mitochondrial function and associated oxidative stress.

In contrast, in fungi and protist species, activation occurs when a low energy status is reached. As

discussed previously, many biotic and abiotic factors are known to limit or abolish the activity of the cytochrome pathway. Under these conditions, the energy status of the cell is reduced and the AMP concentration increases. Indeed, in *U. maydis* cells an inverse relationship between ATP and AMP was found, such that a decrease in ATP, produced by the addition of metabolic inhibitors, was followed by an increase in AMP, indicating that in this fungus the adenylate kinase acts as a nucleotide buffer system, as described in other organisms [64,65]. In addition to this central role, adenylate kinase forms part of a signaling system, sensing the energy status of the cell and modulating the AMP concentration, which in turn regulates the activity of the metabolic machinery. It is remarkable that the regulatory mechanisms of fungal and protist AOX by AMP [66–68] resemble activation of phosphofructokinase I by the same compound [69], suggesting that both systems have evolved as rescue mechanisms that are specifically activated when the cytochrome pathway or mitochondrial metabolism is impaired.

Glycolytic activity in *U. maydis*

We have stressed that despite the economic importance of the corn smut, many aspects of the biology of *U. maydis* remain unstudied, in particular its energy metabolism. The central points of this and our previous studies have focused on mitochondrial metabolism, but in this study it became apparent that glycolysis was significant in this organism. For example, inhibition of glycolysis did not affect ATP content, unless the mitochondrial metabolism was impaired. It is evident that the relative participation of glycolysis in ATP synthesis is closely related to mitochondrial activity, which is consistent with the presence of the Pasteur effect in this organism, and suggests that glycolytic activity could be controlled via phosphofructokinase I, as in many other systems [69].

Conclusion

One of the most interesting questions about the role of AOX in cell physiology is the price the cell pays and the benefits obtained. From this perspective, the relevance of this enzyme in the cell metabolic trends can be understood in terms of a cost/benefit ratio. In *U. maydis* there is a low energy cost associated with the presence of AOX in the cell, because: (a) it is regulated, and therefore is either expressed or activated only under specific conditions (i.e. during cell duplication or when the cytochrome pathway is inhibited); and (b) it makes a small contribution to cell

respiration, so few electrons escape from the main ATP-synthesizing cytochrome pathway. In addition, the presence of AOX represents great benefits because: (a) AOX allows the mitochondrial metabolism to be active under conditions, generated by biotic or abiotic interactions, that may limit the activity of the cytochrome pathway; and (b) as shown previously, AOX activity is essential for redox homeostasis. On this basis, it is clear that the presence of AOX might be valuable to free-living organisms that are exposed to variations in temperature and nutrient availability, among other conditions. In addition, AOX may accomplish some accessory functions [70,71].

Experimental procedures

Cell culture and manipulation

The FB$_2$ strain of wild-type *U. maydis* saprobium monokaryotic cells was cultured, as described previously [72]. Cells were grown at 28 or 34 °C, in YPD medium (yeast extract 1%, glucose 1%, bactopeptone 0.25%, pH 4.7) or in minimal medium (glucose 1%, NH$_4$NO$_3$ 0.15%, salts solution, pH 5), supplemented in some cases with bactopeptone (0–0.6%). Cells were harvested by centrifugation, washed twice with distilled water, and resuspended in KME medium (KCl 120 mM, Mops 10 mM, EGTA 1 mM, MgCl$_2$ 5 mM, KH$_2$PO$_4$ 5 mM, pH 7.0). Cell density was estimated by reading the absorbance at 600 nm (A_{600}). For comparison, one absorbance unit of the harvested cells contains 0.5–0.7 mg of total cell protein.

Oxygen consumption

Oxymetric measurements were performed with intact cells using a Clark-type oxygen electrode, at 25, 28 or 34 °C, measuring respiratory activity with the endogenous substrates. The capacities of AOX and the cytochrome pathway (bc_1 and aa_3 cytochromes) [73] were considered as the uncoupled oxygen consumption rate (PCP 4 μM) in the presence of an inhibitor of the complementary pathway (KCN 1 mM or nOg 2 μM). However, addition of the uncoupler did not affect the AOX capacity.

AOX immunoblotting

Western blots were performed as reported previously [4]. The AOX antibody was kindly donated by D. González-Halphen (IFC, UNAM). Mitochondrial preparations from cells grown in YPD medium were obtained as described previously [4]. For cells grown in minimal media, mitochondrial preparations were obtained by the cell wall lysing method described by Waterfield & Sisler [74], with minor modifications.

Fluorometric measurements

To estimate ROS production, the fluorometric signal of the H$_2$O$_2$ indicator dihydrodichlorofluorescein diacetate was followed [75]. Cells were loaded with 10 μM of the dye, in the presence of KCN and/or nOg in KME medium, either at 28 or 34 °C. After 30 min, cells were washed and resuspended in KME medium, and the fluorescence was read at excitation and emission wavelengths of 488 and 520 nm, at 25 °C. It is important to note that the H$_2$O$_2$ production patterns were similar in cells loaded with dye for 5 or 30 min, but higher loading times gave better signal-to-noise ratios (> 500).

The behavior of the mitochondrial membrane potential in intact cells was studied using the fluorometric probe JC-1 [76]. Cells were loaded for 15 min at 25 °C with 20 μM dye, and washed twice. Fluorescence was read at an excitation wavelength of 490 nm, and emission wavelengths of 527 nm (for the monomeric JC-1, membrane potential insensitive) and 590 nm (for the J-aggregate form of JC-1, membrane potential sensitive), in KME medium at 28 or 34 °C.

Enzyme activities

Enzymatic activities were determined in cytosolic extracts, from cells grown under different culture conditions. Cytosolic extracts were obtained by differential centrifugation. Briefly, cells were broken mechanically with glass beads, and the suspension was centrifuged at 300 *g* for 5 min. The supernatant was centrifuged at 1000 *g* for 10 min, and the resulting supernatant was further centrifuged at 100 000 *g* for 40 min. Glutathione reductase was measured as described previously [77], following the decrease in NADPH concentration at 340 nm. Catalase was determined pollarographically following the increase in O$_2$, after the addition of H$_2$O$_2$ (20 mM). Protein determination was performed using the Biuret method.

Adenine nucleotide determination

ATP, ADP and AMP were determined by the enzymatic methods described by Bergmeyer & Bernt [78]. Glucose was determined with a commercial kit (Spinreact, xxxxxxxxxx), through the production of H$_2$O$_2$ by glucose oxidase, and the oxidation of 4-aminophenazone by peroxidase.

Acknowledgements

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Supplementary material

The following supplementary material is available online:

12 **Fig. S1.** xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

This material is available as part of the online article from http://www.blackwell-synergy.com

3.3 El papel metabólico de la NDH-2 en *Ustilago maydis*

Resumen

Una de las funciones más importantes de la mitocondria es oxidar al NADH que se produce en el citosol. Debido a que la membrana interna mitocondrial es impermeable a esta molécula, se requiere que los electrones sean incorporados a diversos intermediarios carbonados, para que posteriormente sean introducidos a la cadena respiratoria. Los mecanismos encargados de este proceso se conocen como lanzaderas y existe una gran variedad de ellas. Debido a que *Ustilago maydis* no es un organismo fermentador, es posible que la mayoría del NADH citosólico sea oxidado por la mitocondria, por lo cual es importante caracterizar los mecanismos de lanzadera presentes en este hongo. Los datos sugieren que la lanzadera más importante en este organismo es la isoforma externa de la NADH deshidrogenasa alterna, debido a que su actividad resultó muy alta y fue constitutiva. Sin embargo, esta lanzadera puede estar acompañada por otros sistemas, como la lanzadera de glicerol 3-fosfato, y posiblemente por la de malato- oxaloacetato o la de acetaldehído- etanol, aunque estas actividades dependen de las condiciones particulares de cultivo.

Introducción

En una gran cantidad de vías metabólicas se llevan a cabo procesos de transferencia de electrones, en las que se producen moléculas reducidas, como el NADH. Debido a que la concentración intracelular de NAD^+ total ($NADH + NAD^+$) es relativamente pequeña (~1 mM) [1], se requieren procesos que reoxiden al NADH, de manera de se puedan alcanzar estados estacionarios estables, en los cuales la poza de NAD^+ se mantenga oxidada, con una relación $NADH/ NAD^+$ de aproximadamente 1:1000 [1-5], lo cual garantiza la unidireccionalidad de los procesos metabólicos.

Debido a su función oxidativa, la mitocondria posee mecanismos que son capaces de transportar los electrones del NADH citosólico a la matriz mitocondrial, sin que la propia molécula de NADH atraviese la membrana interna, manteniendo así las pozas de NAD^+ separadas [1]. Estos sistemas se conocen como lanzaderas redox, de las cuales las mejor caracterizadas en los mamíferos son la lanzadera de glicerol 3- fosfato y la de aspartato-

malato [1, 6, 7]. En otros organismos eucariontes se encuentran sistemas accesorios, como la lanzadera de acetaldehído-etanol y la de malato-oxaloacetato [1], y también se puede encontrar la lanzadera compuesta por la isoforma externa de la NADH deshidrogenasa alterna (ND2e), la cual no requiere de intermediarios, ya que toma directamente los electrones del NADH citosólico y los transfiere a la molécula de ubiquinona [1, 6, 8, 9]. Recientemente hemos iniciado la caracterización del metabolismo energético de *Ustilago maydis* [10]. A pesar de que no se conocen los sistemas de oxidación del NADH citosólico, es claro que este hongo es un organismo aerobio y no se tiene evidencia de que tenga altas tasas de fermentación [11], por lo cual es posible que la mayoría del NADH citosólico sea tomado por la mitocondria. En este estudio se caracterizaron los mecanismos de lanzadera redox mitocondrial presentes en *U. maydis.*

Material y Métodos

Cultivos celulares

Se utilizó la cepa FB_2 de *U. maydis*, que crece en forma de esporidios unicelulares, saprobios y uninucleados. Las células fueron cultivadas como se reporta en trabajos anteriores [10], en medio YPD (extracto de levadura 1%, glucosa 1%, extracto de bactopeptona 0.25%, pH 4.7) o en medio mínimo (NH_4NO_3 0.3 %, solución de sales, pH 5.0) con etanol (5 mM) o malato (50 mM) como fuentes de carbono, a 28 °C, en agitación continua (250 rpm), durante 24 hrs. Las células se cosecharon por centrifugación y se lavaron dos veces con agua destilada, para ser finalmente resuspendidas en medio KME-MgPi (KCl 120 mM, MOPS 10 mM, EGTA 1 mM, $MgCl_2$ 10 mM, KH_2PO_4 10 mM, pH 7.0), en el que se realizaron los experimentos oximétricos.

Permeabilización de las células

Las células se permeabilizaron con digitonina (0.6 mg AU_{600}^{-1} ml^{-1}), como se describió anteriormente [10], y se lavaron dos veces por centrifugación en medio KME-MgPi.

Consumo de oxígeno

La respiración de las células permeabilizadas se midió polarimétricamente con un electrodo de oxígeno de tipo Clark, a 25 °C, en medio KME-MgPi.

Resultados

Las células permeabilizadas ofrecen un buen sistema para estudiar los procesos en los cuales es de interés considerar el complemento metabólico completo de la célula. Este sistema también es útil para conocer el comportamiento de los sistemas enzimáticos en condiciones en las que la concentración de las proteínas es alta y donde las interacciones macromoleculares se conservan. Además, la cantidad de material biológico que se requieren es pequeña, comparada con la metodología utilizada para obtener preparaciones mitocondriales, y el tiempo invertido también es menor.

En un trabajo anterior se describió la organización de la cadena respiratoria de *U. maydis* con el sistema de células permeabilizadas [10], obteniéndose buenas actividades respiratorias con diversos sustratos mitocondriales. Sin embargo, el mayor inconveniente de esta técnica es que la fosforilación oxidativa se desacopla, por lo cual no es posible realizar estudios más profundos sobre el sistema fosforilante, aunque la membrana interna mitocondrial sigue siendo impermeable a moléculas pequeñas como el NADH.

Los sistemas de lanzaderas redox mitocondriales de *Ustilago maydis*

Para tener una visión general de los mecanismos mitocondriales de oxidación del NADH externo en *U. maydis*, se realizó una búsqueda informática en la base de datos del genoma de este organismo de las enzimas y transportadores que participan en cada una de las lanzaderas (Tabla I). De manera interesante se encontró que *U. maydis* tiene el complemento genético para llevar a cabo todos los procesos de lanzadera descritos.

Enzima o transportador	Clave de acceso	Valor de E	Péptido de tránsito mitocondrial
Lanzadera de Aspartato- Malato			
Malato deshidrogenasa [1]	EAK80785	5×10^{-91}	Si
	EAK85863	6×10^{-48}	No
Aspartato transaminasa [2]	EAK81244	1×10^{-105}	Si
	EAK85536	6×10^{-113}	No
Acarreador aspartato- glutamato [3]	EAK86786	3×10^{-46}	
Acarredor de dicarboxilatos [4]	EAK82283	2×10^{-61}	
Lanzadera de Glicerol 3-fosfato			
cG3PDH [5]	EAK81204	2×10^{-101}	
mG3PDH [6]	EAK82372	0	
Lanzadera de Acetaldehído- Etanol			
Alcohol deshidrogenasa [7]	EAK82646	7×10^{-76}	Si
	EAK83701	5×10^{-66}	No
Aldehído deshidrogenasa [8]	EAK83639	4×10^{-122}	
	EAK84661	6×10^{-119}	
	EAK83677	5×10^{-98}	
	EAK86656	2×10^{-87}	Si
Lanzadera de Malato- Oxaloacetato			
Proteína acarreadora de oxaloacetato [9]	EAK84898	4×10^{-57}	
NDH-2 [10]	EAK83286	1×10^{-94}	Si
	EAK81746	3×10^{-49}	Si
	EAK84847	1×10^{-45}	Si

Tabla I. Marcos abiertos de lectura encontrados en el genoma de *Ustilago maydis* que presuntamente codifican para las enzimas o transportadores de los diversos sistemas de lanzadera redox. 1-6, valor de E encontrado en el análisis de Blast tomando como referencia la malato deshidrogenasa mitocondrial (NP_112413), la aspartato transaminasa citosólica (JT0439), el acarreador de aspartato- glutamato (Q12482), el acarreador de dicarboxilatos (Q06143), la glicerol 3-fosfato deshidrogenasa citosólica (NP_071551) o mitocondrial (CAA55329) de *Rattus norvergicus*. 7-10, valor de E encontrado en el análisis de Blast tomando como referencia la proteína acarreadora de oxaloacetato (CAB52216), la alcohol deshidrogenasa citosólica (P00330), la aldehído deshidrogenasa citosólica (NP_015264) y la ND2i (CAA89160) de *Saccaromyces cerevisiae*.

Para determinar cuáles de estos sistemas son activos, se realizaron experimentos donde se evaluó la velocidad de oxidación de los sustratos de las diversas lanzaderas en el sistema de las células permeabilizadas. Se eligieron tres condiciones de cultivo, el medio YPD, que ha sido ampliamente utilizado en nuestro grupo, y el medio mínimo suplementado ya sea con etanol (ME) o con malato (MM) como fuentes de carbono, con la finalidad de evidenciar la presencia de las dos lanzaderas redox más elusivas, la de acetaldehído- etanol y la de malato- oxaloacetato.

En la figura 1 se muestra que la respiración mitocondrial de las células permeabilizadas que se cultivaron en medio YPD tiene una buena respuesta a la adición de NADH externo, que se inhibe parcialmente con flavona, como se describió anteriormente [10]. La adición de glicerol 3- fosfato también promovió un aumento significativo del consumo de oxígeno, que fue ∼30% menor que con NADH. Esta respuesta también puede ser obtenida al reconstituir a la lanzadera de glicerol 3-fosfato, adicionando fructosa 1,6- bifosfato, aldolasa y NADH, en presencia de flavona, la cual no parece afectar la respiración dependiente de glicerol 3-fosfato (Figura 1B). Sin embargo, el sistema reconstituido presenta una velocidad respiratoria 4-5 veces menor que con glicerol 3-fosfato. De manera interesante, las células cultivadas en MM mostraron una disminución de 3.5 veces en la oxidación del glicerol 3-fosfato, mientras que las células cultivadas en ME no mostraron actividad respiratoria con este sustrato.

Por otro lado, las células cultivadas en ME fueron capaces de oxidar eficientemente al etanol, tanto intramitocondrialmente como en el citosol, mientras que en las células cultivadas en medio YPD y en MM no se encontró respuesta respiratoria con este sustrato (Figura 2).

En las células cultivadas en medio YPD o en MM no se encontró respiración en presencia de aspartato (10 mM), α-cetoglutarato (10 mM) y NADH, los cuales son los sustratos de la lanzadera de aspartato- malato. Sin embargo, la respiración se estimuló al adicionar a la aspartato transaminasa exógena (Figura 3), y se inhibió en presencia de rotenona, que inhibe al complejo I mitocondrial, o de oxamato, que inhibe a la asparato transaminasa. En contraste, en las células cultivadas en ME la respiración no se estimuló con los sustratos de la lanzadera de aspartato- malato, ni con la adición de la aspartato transaminasa.
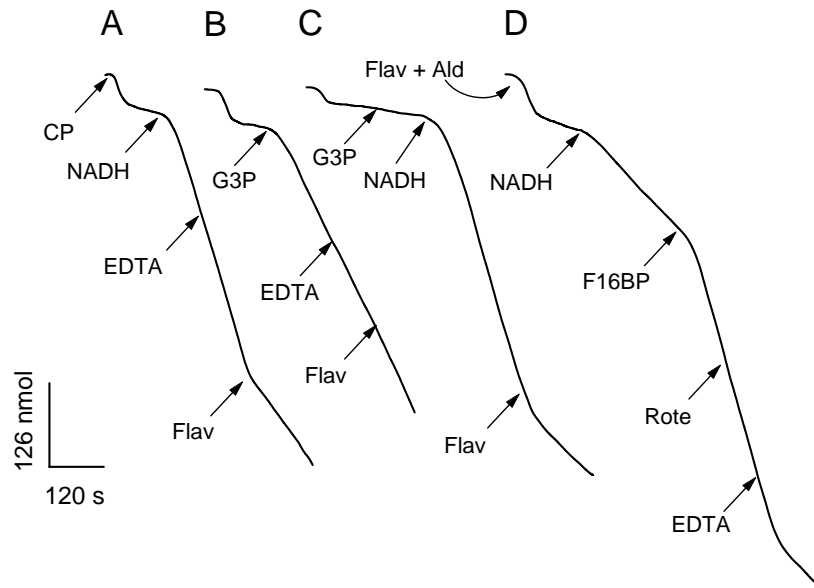
Figura 1. Respiración de las células permeabilizadas (CP) de *Ustilago maydis* cultivadas en medio YPD (A, B y D) o en ME (C). La respiración se estimuló al adicionar NADH (A), glicerol 3-fosfato (B y C) o al reconstituir a la lanzadera de glicerol 3-fosfato (D), con fructosa 1,6-bifosfato (F16BP) y aldolasa (Ald), en presencia de flavona (Flav). En este último caso se adicionó una mayor cantidad de células permeabilizadas (4 UA_{600} ml^{-1}), para obtener velocidades apreciables. En el trazo C se muestra el consumo de glicerol 3-fosfato y de NADH de células permeabilizadas, cultivadas en medio mínimo con etanol.
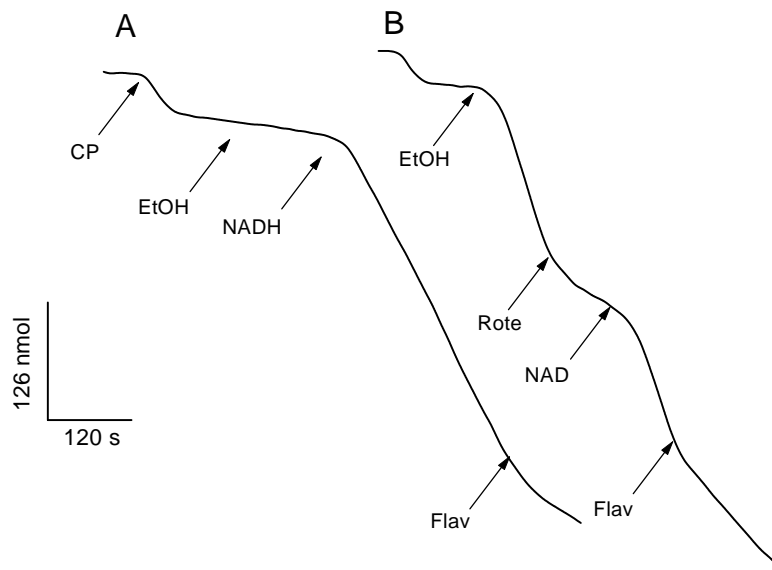
Figura 2. Respiración dependiente de etanol de células permebilizadas cultivadas en medio YPD (A) o en ME (B).
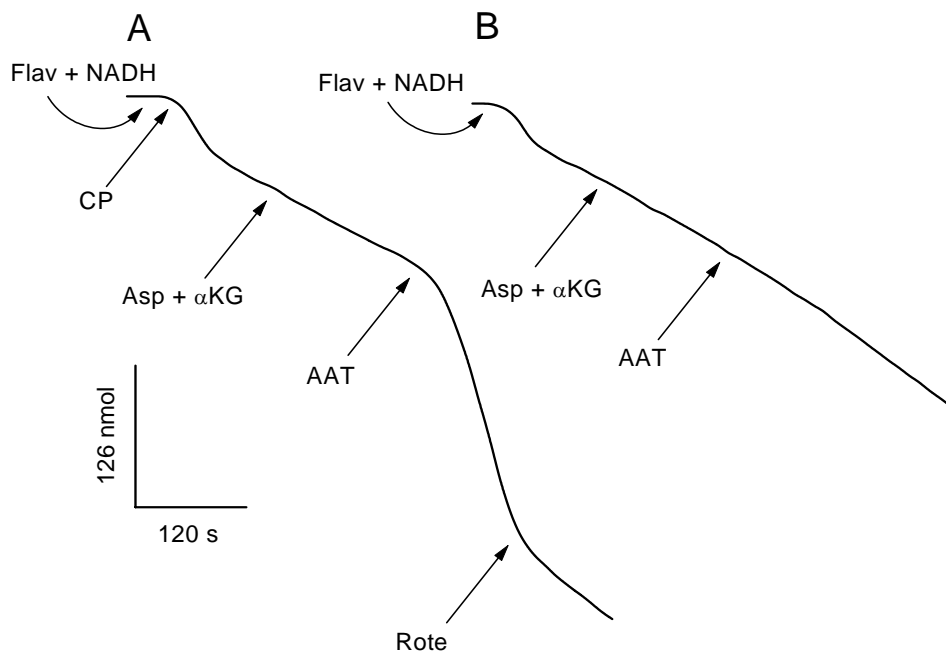
Figura 3. Actividad de la lanzadera de aspartato- malato en células permeabilizadas cultivadas en medio YPD (A) o en ME (B).

Para caracterizar a la lanzadera de malato- oxaloacetato se determinó si la mitocondria de *U. maydis* puede oxidar al malato exógeno y si es capaz de exportar al oxaloacetato que se produce intramitocondrialmente, como lo hacen las mitocondrias de plantas y de algunos hongos [1-3, 5, 6]. En las células cultivadas cultivadas con etanol no se observó una velocidad de oxidación de malato significativa y en las células cultivadas en medio YPD la oxidación de malato se pudo apreciar solamente en concentraciones muy altas (inserto de la Figura 4), posiblemente debido a que la reacción de la malato deshidrogenasa se encuentra desplazada hacia la reducción del oxaloacetato (Keq= 1.5×10^{-5}) [12]. Por otro lado, en las células cultivadas en medio MM la oxidación de este sustrato fue muy eficiente (Figura 4). Además, se encontró evidencia de que la lanzadera de malato- oxaloacetato puede ser activa en estas células, ya que la respiración con NADH externo y succinato se inhibe al adicionar rotenona, y es mayor que la esperada tomando en cuenta la suma de las respiraciones individuales con cada sustrato.
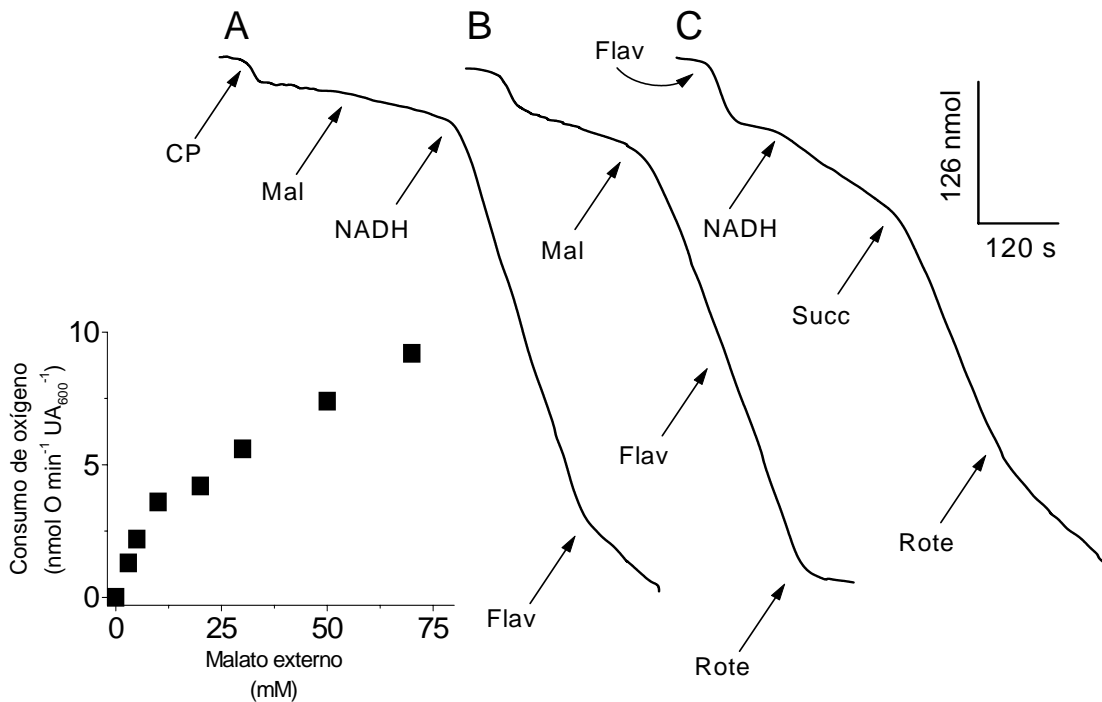
Figura 4. Oxidación de malato externo en células permeabilizadas. A) Células cultivadas en médio YPD. B) y C) Células cultivadas en MM. En el trazo C se adicionó una mayor cantidad de células para obtener velocidades apreciables. En el inserto se muestra la dependencia de la respiración de las células cultivadas en medio YPD con respecto a la concentración de malato externo.

Discusión

En la operación del metabolismo celular se producen activamente equivalentes reductores, en forma de NADH, a través de una gran cantidad de vías metabólicas, de las cuales, la mejor caracterizada es la glucólisis. Sin embargo, otros procesos también son productores de NADH, como la biosíntesis de ácidos grasos y la de algunos aminoácidos [1, 13, 14], así como otras reacciones encaminadas a la duplicación celular. Entre los sistemas que oxidan al NADH que se produce en el citosol se encuentra la fermentación, que en la mayoría de los organismos se lleva a cabo en condiciones anaerobias o microaerofílicas, con excepción de algunos hongos ascomicetos, que la realizan incluso en condiciones aerobias [1, 15]. Sin embargo, la fermentación puede influir negativamente en la economía de la célula, ya que

en este proceso se reducen y expulsan compuestos carbonados, lo cual disminuye el rendimiento celular, definido como la biomasa producida por átomo de carbono utilizado (Ys).

En los organismos aerobios el mecanismo más importante para oxidar al NADH citosólico es la incorporación de los electrones a la cadena respiratoria, para ser donados en última instancia al oxígeno molecular, lo cual representa una doble ventaja con respecto a la fermentación, ya que no se expulsan compuestos carbonados fuera de la célula (por lo que el valor de Ys es alto), y adicionalmente en el propio proceso de oxidación se producen de 3 a 5 moléculas de ATP por cada par de moléculas de NADH oxidadas. Para que la mitocondria pueda tomar estos electrones se requieren los sistemas denominados lanzaderas redox. En algunos tejidos de los mamíferos se ha demostrado que la lanzadera más importante es la de aspartato- malato, posiblemente por ser la que mejor conserva la energía derivada de la oxidación del NADH, debido a que su actividad está vinculada al complejo I mitocondrial. Mientras que, en otros tejidos la lanzadera más importante es la de glicerol 3-fosfato, aunque la razón de esta distribución no se conoce. En otros organismos se aprecia una mayor diversidad de lanzaderas, mostrando sistemas adicionales como son la lanzadera de acetaldehído- etanol (descrita en hongos ascomicetos) [1], la de malato- oxaloacetato (presente primordialmente en plantas) [1-5] y la compuesta por la ND2e (encontrada en una gran cantidad de organismos como hongos, plantas y diversos protistas) [1, 6, 9].

Las lanzaderas redox mitocondriales de *Ustilago maydis*

La NDH-2 y la lanzadera de glicerol 3-fosfato

Aunque se describió que la mitocondria de *U. maydis* presenta al menos una isoforma de ND2e, no se habían descrito otros procesos mitocondriales de tipo lanzadera en este hongo. En el presente trabajo se mostró que además de la ND2e, la lanzadera de glicerol 3-fosfato también se encuentra activa, debido a que las células permeabilizadas pueden oxidar al NADH externo a través de la reducción de la DHAP y la posterior oxidación del glicerol 3-fosfato. Sin embargo, su actividad es sensiblemente menor que la de la ND2e, posiblemente debido a la baja actividad de la glicerol 3-fosfato deshidrogenasa citosólica, ya que la

actividad de este sistema es menor con DHAP y NADH como sustratos, con respecto al glicerol 3-fosfato. En otras condiciones de cultivo se encontró que su actividad es pequeña (MM) o prácticamente nula (ME).

La lanzadera de aspartato- malato

Se demostró que la lanzadera de aspartato- malato no es funcional en ninguna de las condiciones evaluadas, ya que las células permeabilizadas no son capaces de oxidar los sustratos de este sistema (NADH, α-cetoglutarato y aspartato). Los datos indican que la mayor parte de las enzimas de esta lanzadera son activas en las células cultivadas en medio YPD o MM, con excepción de la aspartato transaminasa citosólica, debido a que la respiración se estimula cuando se reconstituye el sistema adicionando exógenamente a esta enzima y se detiene al adicionar su inhibidor (oxamato) [16]. Como cabría esperar, esta actividad respiratoria es completamente sensible a rotenona, ya que en este hongo el complejo I es la única NADH deshidrogenasa interna. En las células cultivadas en ME no se registró una actividad apreciable de esta lanzadera, ni siquiera al adicionar exógenamente a la aspartato transaminasa, lo que sugiere que en esta condición otras enzimas, como la malato deshidrogenasa, presentan una baja actividad.

La lanzadera de acetaldehído- etanol.

Se encontró que la lanzadera de acetaldehído- etanol no es activa en la mayoría de las condiciones probadas, ya que las células permeabilizadas no oxidan a velocidad apreciable al etanol, lo cual es una condición necesaria para que este sistema opere, posiblemente debido a que la alcohol deshidrogenasa mitocondrial no se encuentra activa. Sin embargo, las células cultivadas en ME son capaces de oxidar al etanol, tanto intramitocondrialmente como en el citosol, lo que sugiere que las dos isoformas de la ADH son activas, como en el caso de *Saccharomyces cerevisiae* [15]. Este resultado abre la posibilidad de que en estas condiciones el transporte de equivalentes reductores se podría llevar a través de esta lanzadera. No obstante, la operación de este sistema requiere de que el acetaldehído que se produce intramitocondrialmete se acumule, debido a que el movimiento del par redox

etanol/ acetaldehído a través de la membrana interna mitocondrial no está mediado por transportadores, sino por difusión pasiva. Sin embargo, en el caso de *U. maydis* es posible que el acetaldehído generado intramitocondrialmente no alcance una concentración alta debido a que podría ser oxidado e incorporado al ciclo de Krebs, mediante la acetaldehído deshidrogenasa mitocondrial (Tabla I), la cual presenta una Km hacia el acetaldehído en el intervalo submicromolar [17]. Es importante señalar que el marco abierto de lectura que presuntamente codifica para la aldehído deshidrogenasa mitocondrial de *U. maydis* (EAK86656) presenta una similitud muy alta (valores de E de $1x10^{-93}$ a $1x10^{-99}$) al compararlo con los genes Ald4 (NP_015019.1) y Ald5 (NP_010996.1) de *S. cerevisiae*, que codifican para las aldehído deshidrogenasas mitocondriales inducibles (reprimibles por glucosa) y constitutivas, involucradas en el metabolismo mitocondrial del etanol.

La lanzadera de malato- oxaloacetato

Para que la lanzadera de malato- oxaloacetato lleve a cabo su función se requieren dos condiciones fundamentales: a) que la mitocondria pueda transportar y oxidar al malato exógeno, y b) que pueda exportar al oxaloacetato que se produce internamente. Se mostró que las células cultivadas en ME no oxidan eficientemente al malato, y que la lanzadera de aspartato- malato no se puede reconstituir al adicionar a la aspartato transaminasa, lo cual sugiere que la malato deshidrogenasa citosólica podría tener una actividad pequeña en esta condición. Por otro lado, se determinó que en las células cultivadas en medio YPD las isoformas citosólica y mitocondrial de la malato deshidrogenasa son activas, ya que la lanzadera de aspartato- malato puede ser reconstituida artificialmente. A pesar de ésto, las células permeabilizadas muestran una velocidad de oxidación de malato pequeña, comparada con la respiración obtenida con NADH externo, y las concentraciones de malato requeridas para obtener una buena actividad son muy altas y posiblemente no se alcanzan en el citosol, lo que sugiere que esta lanzadera no contribuye de manera importante a la oxidación del NADH citosólico. Además, es posible que la producción de oxaloacetato y su transporte hacia el citosol (en el caso de que ocurriera) podrían ser muy bajas en presencia de piruvato, porque el oxaloacetato sería dirigido hacia el ciclo de Krebs. Otro factor que podría limitar el funcionamiento de la lanzadera de malato- oxaloacetato puede ser que la

reacción de la malato deshidrogenasa dentro de la mitocondria está dezplazada hacia la formación de malato, lo cual también debería afectar las actividades del ciclo de Krebs y de la lanzadera de aspartato- malato, ya que para alcanzar una concentración de oxaloacetato de 2 μM, la concentración de malato mitocondrial tendría que ser 133 mM, dadas las condiciones reductoras de la matriz mitocondrial (NAD= 0.5 mM y NADH =0.5 mM [4, 5, 24]). Aun en este caso, la concentración de oxaloacetato sería baja comparada con los valores de Km de la citrato sintetasa (Km= 10-20 μM) [18,19] o de la aspartato transaminasa (Km= 30 -60 μM) [20]. La manera en la que el ciclo de Krebs y la lanzadera de aspartato- malato pueden operar bajo estas circunstancias es a través de la canalización enzimática entre la malato deshidrogenasa mitocondrial con la citrato sintetasa [21, 22], o con la aspartato transaminasa mitocondrial [22, 23].

A pesar de estas limitaciones, se ha reportado que en la mitocondria de las plantas la lanzadera de malato- oxaloacetato es el mecanismo de oxidación del NADH citosólico más importante cuando la concentración de NADH externo es baja. Esto sugiere que esta lanzadera es activa en las plantas gracias a un proceso de canalización enzimática, posiblemente entre la malato deshidrogenasa y el acarreador de oxaloacetato (Km= 160 μM [25]), aunque este proceso no ha sido caracterizado. De acuerdo con esta propuesta, en *U. maydis* la malato deshidrogenasa y el acarreador de oxaloacetato no deberían encontrase asociados, impidiendo el funcionamiento de la lanzadera de malato- oxaloacetato.

De manera interesante, las células cultivadas en MM oxidan al malato a una velocidad importante, lo que de hecho no es consistente con lo discutido sobre el metabolismo de este sustrato en la mitocondria. Sin embargo, este resultado sugiere que en esta condición de cultivo otras enzimas podrían ser activas, como la enzima málica, que podría alimentar a la mitocondria con piruvato. Un resultado sumamente interesante fue que la respiración con NADH externo y succinato se puede inhibir por rotenona, lo cual indica que cuando los dos sustratos están presentes, una fracción importante de los electrones del NADH son introducidos a la mitocondria, donde los toma el complejo I. Una posibilidad para explicar este fenómeno de transporte podría ser que durante la oxidación del succinato se produce oxaloacetato, que sale de la mitocondria y es reducido por la malato deshidrogenasa citosólica, y en forma de malato es introducido y oxidado en la matriz mitocondrial.

Conclusión

La ND2e parece ser la lanzadera redox mitocondrial más importante en *Ustilago maydis*, principalmente por mostrar una actividad alta y constitutiva. Sin embargo, puede estar acompañada por otros sistemas, como la lanzadera de glicerol 3-fosfato, y bajo condiciones particulares de cultivo también pueden estar presentes la lanzadera de malato- oxaloacetato y posiblemente la de acetaldehído- etanol. La caracterización cinética de los diversos procesos mitocondriales de oxidación del NADH externo y la caracterización del contenido intracelular de piridin nucleótidos nos permitiran entender con mayor profundidad el metabolismo del NADH de este organismo.

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4. DISCUSIÓN GENERAL

Y RESULTADOS ADICIONALES

"J- Cíñete, pues, los lomos como hombre valeroso. Yo te preguntaré y tú me harás saber.
H- Pregunta.
J- ¿Has pisado tú por las honduras recónditas del abismo?
H- No, pero he entrado al imperio corrosivo y sin límites de la injusticia.
J- ¿Sabes tú cuándo paren las cabras monteses?
H- No, pero sé cuando el arzobispo bendice el puñal y la pólvora.
J- Y en cuanto a las tinieblas... ¿dónde está el lugar de las tinieblas?
H- En la mirada y en el pensamiento de los hombres... ¡Tuya es la luz!
J- ¿Y has penetrado tú hasta los manantiales del mar?
H- No, pero he llegado hasta el venero profundo de las lágrimas... ¡Mío es el llanto!
H-Y ahora pregunta el hombre, ahora pregunto yo... y tú me harás saber:
¿Para qué sirve el llanto?
Sino es para comprarte la luz... ¿para qué sirve el llanto?
¿Por qué hemos aprendido a llorar?
El llanto ¿no es más que la baba de un gusano?
¿Lloramos sólo porque tú has apostado con Satán?
Nuestra lepra, esta lepra de ahora ¿ha salido también del gran cubilete de tus dados?
Ya sé, ya sé que somos tan sólo una jugada tirada sobre la mesa verde de tu gloria; ya sé, ya sé que apuestas ahí arriba con el diablo, a
la luz y a la sombra, como al rojo y al negro en un garito...
Que ahora ha salido el negro, que ha triunfado la sombra, que Satán te ha vencido.
¿Y yo no soy más que una ficha, una moneda, una res, un esclavo...
el objeto que se apuesta, lo que va de un paño a otro paño, de una bolsa a otra bolsa?
¡Oh, no!
Yo puedo gritar, yo puedo llorar,
yo puedo ofrecer mi llanto, todo mi llanto por la luz... ¡por una gota de luz!
Sí, sí.
Yo puedo llorar
y gritar
y patear
y denunciar la trampa.
Y aunque sueltes sobre mi boca todos los ladridos del trueno, me oirás.
Y aunque arrojes sobre las cuencas de mis ojos las lluvias y los mares, la amargura de mis lágrimas te llegará hasta la lengua.
¡Tuya es la luz!... ¡pero el llanto es mío!"
León Felipe, 1943.

Ustilago maydis es un hongo patógeno que produce la enfermedad denominada carbón del maíz, la cual causa pérdidas millonarias en el cultivo de algunas variedades [8]. Este hongo está emparentado con otros patógenos de especies económicamente importantes, como la caña de azúcar, el sorgo, la avena, el arroz y diversas plantas de ornato [1, 2, 8-11], y de hecho, se considera que los ustilaginomicetos son los hongos más dañinos que existen para la agricultura.

Debido a su importancia comercial como plagas, han sido estudiados con gran profundidad, en particular *U. maydis*, del que se conocen los mecanismos moleculares que controlan el cambio dimórfico, los mecanismos de patogenicidad y los procesos de regulación genética, entre otros [1, 2, 9]. Sin embargo, la mayoría de los estudios se han enfocado en entender ciertos aspectos puntuales de la biología de *U. maydis*, mientras que otros campos han sido prácticamente olvidados, como el metabolismo energético e intermediario. De hecho, hasta la aparición de nuestras aportaciones se habían publicado sólo cinco trabajos, en donde se estudiaron someramente algunos aspectos del funcionamiento de la mitocondria de este organismo [14-18], mientras que otros procesos, como la glucólisis o el ciclo de Krebs, por mencionar algunos, son totalmente desconocidos. En el presente trabajo se abordaron tres líneas principales de investigación. La primera fue el estudio de la organización de la cadena transportadora de electrones mitocondrial de este organismo.

4.1 La organización de la cadena respiratoria de *Ustilago maydis*

Mediante un enfoque funcional (usando sustratos o inhibidores específicos) se determinó que la cadena transportadora de electrones mitocondrial de *U. maydis* está compuesta por los cuatro complejos respiratorios clásicos, que son: la NADH: ubiquinona oxidorreductasa sensible a rotenona (complejo I), la succinato: ubiquinona oxidorreductasa (complejo II), la ubiquinol: citocromo c reductasa (complejo III) y la citocromo c oxidasa (complejo IV).

4.1.1 La AOX

Al caracterizar la respuesta respiratoria hacia diversos inhibidores metabólicos, se determinó que una parte significativa de la respiración celular (20%) fue insensible a los

inhibidores de los complejos III y IV (antimicina y cianuro), lo que indicaba la presencia de una oxidasa terminal accesoria a la vía citocrómica, a nivel de la poza de ubiquinona. Se encontró un número importante de candidatos para esta oxidasa terminal y afortunadamente la mayoría de las ubiquinol oxidasas o citocromo c oxidasas se encuentran restringidas al dominio de las eubacterias [53-55], lo cual redujo las posibilidades a la AOX, ubicua en los eucariontes [60, 62], y la vía paralela de citocromos, descrita en *Candida parasilopsis* [63]. Para discernir entre estos dos candidatos se realizaron estudios con diversos inhibidores de la AOX, como el ácido salicilhidroxámico (SHAM) o el n-octigalato [64], y se determinó que la respiración resistente a cianuro o antimicina fue más del 95% sensible a estos inhibidores, lo que indica que la AOX es activa en este hongo. Para confirmar este resultado se realizó una inmunodetección de tipo Western blot y se encontró una banda de peso molecular semejante al reportado para otras AOX (32.5 kDa) [65-68]. Este resultado nos pareció interesante, ya que el análisis comparativo de la secuencias (Figura 13) muestra que la AOX de *U. maydis* presenta una inserción en la parte central, que no se encuentra conservada. Esto nos llevó a pensar que esta sección podría corresponder a un intrón, y de hecho al eliminarla se predice un peso molecular cercano al obtenido por electroforésis. Sin embargo, los algoritmos disponibles para predecir intrones no reconocen a esta región como un segmento no codificante. Además, el gen de la AOX se ubica dentro de un conjunto de secuencias de tipo EST (expressed sequence tag), provenientes de mRNA, lo que sugiere que el gen no debería presentar intrones. Estudios posteriores serán necesarios para esclarecer este punto.

4.1.1.1 Los mecanismos de regulación de la AOX

Como primer acercamiento para entender la participación de la AOX en el metabolismo celular, se estudiaron sus mecanismos de regulación. Como antecedentes, se ha descrito que la AOX de las plantas tiene dos vías principales de activación, por agentes reductores, como ditiotreitol (DTT), glutatión reducido o tiorredoxina [69-72], y por α-cetoácidos, especialmente por piruvato [69, 70, 73]. La AOX en su forma inactiva es un homodímero estabilizado a través de dos puentes disulfuro intercatenarios, que están formados a su vez por dos residuos de cisteína bien conservados

```
NeuCra        ----MN--TP KVNILHAPG- ---QAAQLSR ALISTCHTRP LLLAGSRVAT SLHPTQTNLS
CanAlb        ----MIGLST YRNLPTLLT- ---TTTVIST ALRSKQLLRF TTTTSTKSRS STSTAATTVG
UstMay        ----MYVSTP IVHVMTSTAG SVAGRNAVSF AVLARTHQHP YNALPSSSRR AFSTSPFHLK
OrySat        MSSRMAGSAI LRHVGGVRLF TASATSPAAA AAAAARPFLA GGEAVPGVWG LRLMSTSSVA
NicTab        ---------- ----MWVRHF PVMGPRSAST VALNDK---- ---------- ---QHDKKVE
AraTha        ----MSYRSI YR---TLRPV LSSSVQSSGL GIGGFRGHLI SHLPN----- -VRLLSSDTS

NeuCra        -SPSPR---- --NFST--TS VTRLKDFFPA KETAYIR--- --------QT PPAWPHHGWT
CanAlb        -NSNPKSPID EDNLEKPGTI PTKHKPFNIQ TEVYNKAGIE ANDDDK-FLT KPTYRHEDFT
UstMay        PAVQAKDRPG SPGLKHPDGS YLLFHPIYSE HELDSVKVVH RESKTFGDKV ARAMCTAART
OrySat        STEAAAKAEA KKADAEKEVV VNSYWGIEQS KKLVREDGT- -------EWK WSCFRPWETY
NicTab        NGGAAASGGG ---DGGDEKS VVSYWGVPPS K-VTKEDGT- -------EWK WNCFRPWETY
AraTha        SPVSGNNQPE NPIRTADGKV ISTYWGIPPT K-ITKPDGS- -------AWK WNCFQPWDSY

NeuCra        EEEMTSVVPE HRK------- ---------P ETVGDWLAWK LVRICRWATD IATGIRPEQQ
CanAlb        EAGVYRVHVT HRP------- ---------P RTIGDKISCY GTLFFRKCFD LVTGYAVP--
UstMay        VFDMATRYPD HNGKKFPPLK RDVEKTVGLP SHLAKQVERL KEQTKDASVN AVAGSATAAA
OrySat        TADTSIDLTK HHV------- ---------P KTLLDKIAYW TVKSLRFPTD IFF-------
NicTab        KADLSIDLTK HHA------- ---------P TTFLDKFAYW TVKALRYPTD IFF-------
AraTha        KPDVSIDVTK HHK------- ---------P SNFTDKFAYW TVQTLKIPVQ LFF-------

NeuCra        VDKHHPTTAT S--------- ---------- ---------- ----ADKPLT EAQWLVRFIF
CanAlb        -DPDKPDQYK G--------- ---------- ---------- ----TRWEMT EEKWMTRCIF
UstMay        ATTTSQSSTT SPTPTATKVV NQTGADLQEL TSNDGAMTLQ EMRAKGLSFG PDGWLNRMIF
OrySat        ---------- ---------- ---------- ---------- ---------- QRRYGCRAMM
NicTab        ---------- ---------- ---------- ---------- ---------- QRRYGCRAMM
AraTha        ---------- ---------- ---------- ---------- ---------- QRKHMCHAML

NeuCra        LESIAGVPGM VAGMLRHLHS LRRLKRDNGW IETLLEESYN ERMHLLTFMK MC-EPGLLMK
CanAlb        LESIAGVPGS VAGFVRHLHS LRMLTRDKAW IETLHDEAYN ERMHLLTFIK IG-KPSWFTR
UstMay        LESIAGVPGM VAATCRHLQS LRLMRRDKGW IHTMLEDAEN ERMHLLVALH LSGKPGLIAR
OrySat        LETVAAVPGM VGGMLLHLRS LRRFEQSGGW IRTLLEEAEN ERMHLMTFME VA-NPKWYER
NicTab        LETVAAVPGM VGGMLLHCKS LRRFEQSGGW IKALLEEAEN ERMHLMTFME VA-KPNWYER
AraTha        LETVAAVPGM VGGMLLHLKS LRRFEHSGGW IKALLEEAEN ERMHLMTFIE LS-QPKWYER

NeuCra        TLILGAQGVF FNAMFLSYLI SPKITHRFVG YLEEEAVHTY TRCIREIEEG -HLPKWSDEK
CanAlb        SIIYIGQGVF TNIFFLVYLM NPRYCHRFVG YLEEEAVRTY THLIDELDDP NKLPDF--QK
UstMay        TFVLLAQGVF YNFFFIFYLL SPRVAHRFVG VLEEEAVLTY SLILEDLKEG -RLPEW--ED
OrySat        ALVITVQGVF FNAYFLGYLL SPKFAHRVVG YLEEEAIHSY TEFLKDLEAG ------KIDN
NicTab        ALVFAVQGVF INAYFVTYLL SPKLAXRIVG YLEEEAIHSY TEFLKELDKG ------NIEN
AraTha        AIVFTVQGVF FNAYFLAYVI SPKLAHRITG YLEEEAVNSY TEFLKDIDAG ------KFEN

NeuCra        FEIPEMAVRY WRMPEGKRTM KDLIHYIRAD EAVHRGVNHT LSNLDQKEDP NPFVSDYKEG
CanAlb        LPIPNIAVQY WPELTPESSF VDLILRIRAD EAKHREINHT FANLEQWQDR NPFALKIKDS
UstMay        VPAPEIAKQY WQLG-DEAML VDVIRAIRAD EATHRHINHT FASLNS-DDP NPFALREPPA
OrySat        VPAPAIAIDY WRLP-ANATL KDVVTVVRAD EAHHRDVNHF ASDIHYQGME LKQTP-----
NicTab        VPAPAIAIDY WRLP-KDSTL RDVVLVVRAD EAHHRDVNHF APDIHYQGQQ LKDSP-----
AraTha        SPAPAIAIDY WRLP-KDATL RDVVYVIRAD EAHHRDINHY ASDIQFKGHE LKEAP-----

NeuCra        EGGRRPVNP- ALKPTGFERA EVIG------ -------
CanAlb        DKPQPNYNLD VTRPQGWERK DLYL------ -------
UstMay        KMRAETYGLE RDEALEWAKG KDLENGSDAA TAAEKTA
OrySat        ---------- --APIGYH-- ---------- -------
NicTab        ---------- --APIGYH-- ---------- -------
AraTha        ---------- --APIGYH-- ---------- -------
```

Figura 13. Alineamiento de las secuencias de AOX. En fondo negro se resaltaron los residuos necesarios para la unión del centro binuclear de Fe, en gris los residuos de cisteína involucrados en la activación por α-cetoácidos y por agentes reductores, en negritas se resaltaron las regiones de la AOX de *U. maydis* que no tienen regiones homólogas. NeuCra, *Neurospora crassa*; CanAlb, *Candida albicans*; UstMay, *Ustilago maydis*; OrySat, *Oryza sativa*; NicTab, *Nicotiana tabacum*; AraTha, *Arabidopsis thaliana*.

El tratamiento con agentes reductores promueve la ruptura de los puentes disulfuro y la activación parcial de la enzima. En su forma reducida, el residuo de cisteína más próximo al amino teminal reacciona covalentemente con el α- cetoácido, formando un tiohemiacetal, con lo cual la AOX se activa por completo [69-73] (Figura 14).
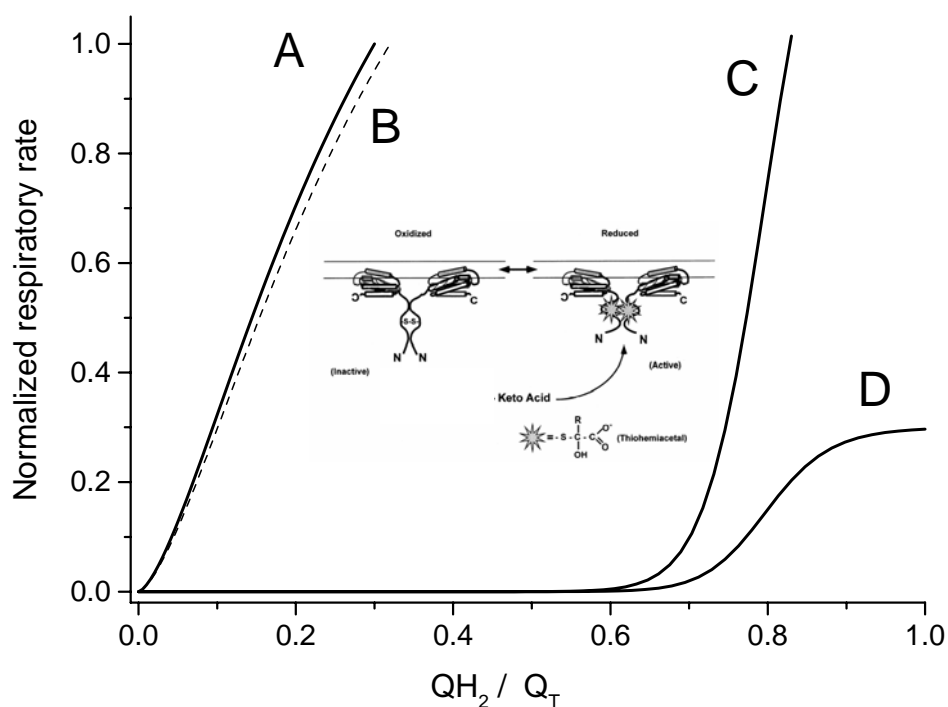
Figura 14. Activación de la AOX de plantas por el tratamiento reductor y por piruvato. Actividad de la vía citocrómica (A) y de la AOX en presencia de DTT y piruvato (B), en presencia de DTT (C) y en ausencia de activadores (D). Tomado de Millar *et al.* (73)

Por otro lado, se ha descrito que la AOX de los hongos y protistas (incluyendo algas clorofíceas) no se activa por el tratamiento reductor o por los α-cetoácidos, ya que los residuos de cisteína involucrados en este proceso de regulación no se encuentran presentes. En cambio, pueden ser estimuladas por mononucleótidos púricos, especialmente por AMP y GMP [74, 75]. Este proceso es particularmente interesante, ya que la AOX es una proteína periférica orientada hacia la matriz mitocondrial que no presenta segmentos transmembranales [76], lo cual implica que el AMP o el GMP se transportan al interior de la matriz mitocondrial, o que su efecto podría ser indirecto, activando a otras proteínas que

a la postre modulan la actividad de la AOX, aunque el mecanismo de activación no está descrito.

Uno de los mayores contratiempos metodológicos que se tuvieron en las primeras etapas del proyecto fue la dificultad para obtener preparaciones mitocondriales activas, por lo que se decidió trabajar con el sistema de células permeabilizadas, el cual ofrece una gran cantidad de ventajas para estudiar la función mitocondrial, aunque encontramos dos grandes desventajas: que la fosforilación oxidativa se desacopla y que la actividad de la AOX se pierde. Para prevenir la inactivación de la AOX se probó el efecto protector de activadores, como el AMP, el DTT y el piruvato. Concordando con las características regulatorias de las AOX de hongos, el piruvato no mostró un efecto importante, mientras que el AMP protegió significativamente la actividad de esta enzima. Sin embargo, se encontró que el tratamiento con DTT también protegió la actividad, mostrando un efecto aditivo con la protección que produce el AMP, lo que sugiere que los dos agentes tienen mecanismos de acción independientes. Para explicar el efecto del DTT se compararon las secuencias de las AOX de diversos organismos, con el fin de verificar si en la AOX de *U. maydis* se encontraban los dos residuos de cisteína regulatorias de las plantas, pero como ocurre con otras AOX de hongos, estos residuos no están presentes (Figura 13). Con los datos presentados en el trabajo no se puede ofrecer una explicación con respecto al mecanismo de protección del DTT, aunque es posible que su efecto sea debido a que mantiene en un estado reducido a las pozas de glutatión, tiorredoxina o de ubiquinona, dado su alto potencial reductor (-350 mV) [77], lo cual podría promover una protección mediada por el sustrato (ubiquinol) o por proteínas que dependan de otros intermediarios reducidos.

4.1.1.2 Regulación a largo plazo de la AOX

Uno de los procedimientos más utilizados para caracterizar los mecanismos de regulación a largo plazo de la AOX se basa en el cultivo de los microorganismos en presencia de los inhibidores del complejo III o IV [4, 78-81]. En *U. maydis*, la adición de antimicina o cianuro al medio de cultivo promovió una disminución de 4 veces en la actividad de la vía citocrómica y un incremento de 5-6 veces en la actividad de la AOX. Es importante aclarar que la actividad de la vía citocrómica no fue correctamente determinada, como se señalaría

en el trabajo posterior, ya que lo que se reportó originalmente fue la respiración sensible a cianuro, y no la insensible a n-octilgalato (o SHAM) y sensible a cianuro, aunque cuantitativamente la diferencia no es sustancial.

Otro de los efectos que se observaron, fue un incremento de 6-7 veces en el consumo de oxígeno no mitocondrial (insensible a los inhibidores de las oxidasas terminales), lo que puede ser un indicador del impacto que tiene la inhibición de la vía citocrómica en la producción de especies reactivas de oxígeno (ROS) y su efecto en las enzimas que manejan el estrés oxidativo. Llama la atención que los procesos que se han asociado con el consumo de oxígeno no mitocondrial están determinados por las actividades de diversas enzimas que producen activamente ROS, como la xantina oxidasa, el citocromo p450, la urato oxidasa, la D- aminoácido oxidasa, la acil- CoA oxidasa o la oxalato oxidasa [82]. Es posible que en esta condición de cultivo la célula haya adaptado su metabolismo con el fin de producir ROS por vías no mitocondriales, ya que la producción mitocondrial de especies reactivas de oxígeno podría ser pequeña, puesto que la mayoría de los electrones deberían fluir a través de la AOX, y se ha comprobado que a pesar de que este tipo de moléculas tienen efectos dañinos sobre las células, también son importantes como mensajeros intra e intercelulares [83, 84].

4.1.2 La NDH-2

En el trabajo de Ziogas *et al.* [18] se determinó que las preparaciones mitocondriales de *U. maydis* tenían una actividad respiratoria considerable con NADH externo, que era parcialmente sensible a la adición de rotenona, lo que indica que las mitocondrias que purificaron estaban rotas, de manera que el NADH externo podía ser oxidado por el complejo I, pero también por una NDH-2 de ubicación desconocida.

4.1.2.1 La isoforma mitocondrial de NDH-2 en *Ustilago maydis*.

Al examinar los patrones obtenidos con los inhibidores de las distintas deshidrogenasas, se determinó que la respiración dependiente de NADH exógeno no se inhibe con rotenona (inhibidor del complejo I), sino con flavona (inhibidor de la NDH-2), mientras que la

respiración con piruvato y malato (que produce NADH intramitocondrial) es completamente sensible a rotenona e insensible a flavona. Estos datos indican que a pesar de que la mitocondria se desacopla durante la permeabilización, la membrana interna sigue siendo impermeable a moléculas pequeñas como el NADH, y de hecho, gracias a esta propiedad se pudo identificar que la oxidación del NADH externo se lleva acabo a través de la isoforma externa de la NDH-2, que es insensible a rotenona, pero se puede inhibir parcialmente con flavona, y que la oxidación del NADH interno se realiza únicamente a través del complejo I, ya que la respiración con malato y piruvato es totalmente sensible a rotenona y es resistente a flavona.

Esta distribución se ha reportado en pocos organismos, de hecho el único ejemplo es *Yarrowia lypolitica* [85], y en la mayoría de los casos se reconoce que la isoforma externa es acompañada por las isoformas internas o por las sensibles a Ca^{++} [59]. Sin embargo, ésto sugiere que *U. maydis* se ha adaptado para que la oxidación del NADH interno, proveniente del ciclo de Krebs, se lleve a cabo únicamente por el complejo I, mientras que la oxidación del NADH citosólico, proveniente de la glucólisis, se realice por la isoforma externa de la NDH-2, como se discutirá más adelante.

4.1.2.2 Las entidades macromoleculares mitocondriales con actividad de NADH deshidrogenasa

De acuerdo con la interpretación anterior, se esperaría encontrar dos entidades con masas moleculares distintas (MW_{CI}= 750-900 kDa, MW_{NDH-2}= 30-70 kDa) capaces de oxidar al NADH. Para probar esta hipótesis se realizaron ensayos enzimáticos de NADH: tetrazolio oxidorreductasa en geles de poliacrilamida en condiciones nativas, mediante la técnica de gel azul de Schägger *et al.* [86]. Se encontraron tres bandas con actividad, dos de alto peso molecular, de 650 y 580 kDa, y una con alta movilidad electroforética, de 84 kDa. La banda de bajo peso molecular posiblemente corresponde a la especie monomérica de la NDH-2, y sería uno de los pocos ejemplos reportados en los que esta enzima puede ser activa como monómero [87]. Sin embargo, en los ensayos de filtración molecular en HLPC que se hicieron para purificar a la NDH-2 de *U. maydis*, se determinó que la proteína soluble migra alrededor de los 110 kDa, lo que correspondería al dímero. La banda de 580

kDa podría corresponder al complejo I, ya que en esta sección del gel se observa una gran cantidad de proteína y el peso molecular concuerda con el reportado para el complejo I de *N. crassa* [88]. La banda de 650 kDa podría ser una subpoblación del complejo I con otras proteínas asociadas, que se pudieron haber perdido en la población de 580 kDa, debido al proceso de solubilización. Sin embargo, esta actividad también podría estar dada por un agregado macromolecular de la NDH-2, como el reportado en la mitocondria de *S. cerevisiae* [89], donde las isoformas interna y externas de la NDH-2 se oligomerizan con otras enzimas, como la malato deshidrogenasa, la citrato sintetasa, la succinato deshidrogenasa, la fumarasa, la glicerol 3-fosfato deshidrogenasa mitocondrial, las L y D-lactato deshidrogenasas, entre otras, produciendo un complejo de aproximadamente 480 kDa, que podría participar en procesos de canalización metabólica de oxaloacetato, NADH y ubiquinona, lo cual incrementa la actividad metabólica al facilitar la interacción de las enzimas con sus sustratos [90- 92].

4.1.2.3 Las isoformas de NDH-2 de *Ustilago maydis*

Para identificar a la isoforma de la NDH-2 que es activa en *U. maydis* se realizó una búsqueda de marcos de lectura abierta similares a la NDH-2 interna de *S. cerevisiae* (scNDI; CAA89160) en el genoma de este organismo. Se encontraron tres secuencias (Tabla II) que presentan los motivos característicos de esta familia, como los sitios de unión del NADH y del FAD, o el residuo de triptofano conservado, entre otros (Figura 15).

	Clave de Acceso	% de Similitud vs scNDI	valor de E	Ubicación
umND2e	EAK83286	58	$1x10^{-94}$	Externa
umCa-NDH2	EAK84847	54	$3x10^{-49}$	Externa, Sensible a Ca^{++}
umND2i	EAK81746	45	$1x10^{-45}$	Interna

Tabla II. Marcos de lectura abierta de *U. maydis* correspondientes a NDH-2. La ubicación submitocondrial hipotética de las tres isoformas se predijo a partir del análisis de similitud mostrado en la figura 16.

Sitio de unión del FAD

```
umNDI     ----RKQRLV VLGTGWGGYA FLKSLSYARF DVKVISPTTS FSFTPLLAQA SCATLDFRSV
umNDE     PQDPSKKTIV VLGSGWGATS LLKNIDTEEY NVVVISPHNY FLFTPLLPSV TVGTLDGRSI
scNDI     PQHSDKPNVL ILGSCWGAIS FLKHIDTKKY NVSIISPRSY FLFTPLLPSA PVGTVDEKSI
atNDECa   KEEHKKKKVV VLGTGWAGIS FLKDLDITSY DVQVVSPQNY FAFTPLLPSV TCGTVEARSI
stNDECa   QPESKKKRVV VLGTGWGGTS FLKDVDISSY DVQVVSPRNY FAFTPLLPSV TCGTVEARSI
cnNDECa   RAMRGKPRLV IVGGGWGAVS LIQSLPAHAY NVTLISPQTY FAFTPLLPSA CVGTIEPRSL
umNDECa   KKLANKERLV IVGGGWAAVG LLKSLDPEKY NVTLISPNNY YLFNPLLPSA AVGTVEPRSL
ncNDECa   KKHKEKPRLV ILGGGWGSVA LLKELNPDDY HVTVVSPANY FLFTPMLPSA TVGTLELKSL
ecNDH2    -MTTPLKKIV IVGGAGGLE MATQLGHKKA KITLVDRNHS HLWKPLLHEV ATGSLDEG—
```

Sitio de unión del NADH

```
umNDI     QEHQLRRLLS FVVVGGGPTG SEFAAELHDL INDELSRLYP -NVCAYATVR LLDAGSTILS
umNDE     SEEEIDRLLH MVVVGGGPTG IEYAAELRDF VESDLIRWYP -EVANKLRVT LVEALPNILP
scNDI     GDPERRRLLS IVVVGGGPTG VEAAGELQDY VHQDLRKFLP -ALAEEVQIH LVEALPIVLN
atNDECa   TEEQRRRKLH FVIVGGGPTG VEFAAELHDF IIEDITKIYP -SVKELVKIT LIQSGDHILN
stNDECa   SEEERRTNLH FVIVGGGPTG VEFAAELHDY VYEDLVKIYP -SVKDFVKIT VIQSGDHILN
cnNDECa   TPDERKKLLS FVVCGGGPTG VEFAAELADM MAEDVLKYYP KILSSEVEVT VVQSRDHILN
umNDECa   TEEERKRLLS FVVCGGGPTG VETAAEISDM INEDVFDYFP KVLRAQAQVH LIQSREHILN
ncNDECa   SDEERKRLLS FVVCGGGPTG VEFAAELFDL LNEDLTLHFP RLLRNEISVH LIQSRDHILN
ecNDH2    ANLGANGKVN IAIVGGGATG VELSAELHNA VKQLHSYGYK GLTNEALNVT LVEAGERILP
```

Motivos de tipo mano EF

Sección amino terminal: Motivo conservado en plantas

```
umNDI     NLVIHPS--HPNPG-------------ANVLNPAADDS--------------------
umNDE     RLKGAEDS-------------------------------------------------
scNDI     QVKGSNN--------------------------------------------------
atNDECa   QVTGCEN--VYAVGDCASIAQRKILGDIANIFKAADADNSGTLTMEELEGVVDDIIVRYP
stNDECa   RVKGCSN--VYALGDCASVDQHKVMEDISTIFEAADKDDSGTLSVEEFRDVLEDIIIRYP
cnNDECa   RVQGAPQGSVYALGDSATVQTN-LMNDLYNLWD--KFDINKDGN---IDYEEWQEMVKYI
umNDECa   RVKGAPLGSMYALGDASTIDTR-LIDQLYDFVDRYDKDKDGKLSYSEFET-----FAQAI
ncNDECa   RLNGTPLGDVYAIGDCSTIQNN-VADHIITFLRNLAWKHGKDPESLELHFSDWRDVAQQI
```

Sección carboxilo terminal: Motivo conservado en hongos

```
umNDI     --------------------HMGSPSQPPTPLDNVFALGDCSASPDALPATAQVASQQG
umNDE     ---------------------------------IFALGDATATQFAPTAQAASQQG
scNDI     ----------------------------------IFAIGDNAFAGLPPTAQVAHQEA
atNDECa   QVELYLKSKHMRHINDLLADSEGNARKEVDIEAFKLALSEADSQMKTLPATAQVAAQQG
stNDECa   QVDLYLKNKHLLEAKDLFRDSEGNEREEVDIEGFKLALSHVDSQMKSLPATAQVAAQQG
cnNDECa   KKKHPLAHRSLTKMRAVFEEFDRDHDEKLTLNEVAELFAKLSKKVTSYPATAQVASQQG
umNDECa   RRKFPIASKHFIKLREVFDQYDVDQDGQLNLNEIANVLIETGNKMTALPATAQVAAQQG
ncNDECa   KKRFPQATAHLKRLDKLFEEYDKDQNGTLDFGELRELLKQIDSKLTSLPATAQRAHQQG
ecNDH2    ----------------------------IYAIGDCASCPRPEGGFVPPRAQAAHQMA
```

Figura 15. Alineamiento de secuencias de NDH-2. NDE, isoforma externa de la NDH-2; NDECa, isoforma externa sensible a calcio de la NDH-2; NDI, isoforma interna de la NDH-2. um, *Ustilago maydis*; nc, *Neurospora crassa*; sc, *Saccharomyces cerevisiae*; at, *Arabidopsis thaliana*; st, *Solanum tuberosum*; cn, *Cryptococcus neoformans*; ec, *Escherichia coli*. umNDE (EAK83286), ncNDECa (EAA32649), umNDI (EAK81746), scNDI (CAA89160), atNDECa (NM_118962b), stNDECa (AJ2455862), cnNDECa (EAL19455.1), umNDECa (EAK84847), ecNDH2 (NP_415627).

Una de estas secuencias presenta una sección con dos elementos semejantes a motivos de unión de Ca^{++} de tipo mano EF (Figura 15), como los descritos en otras NDH-2 externas, en particular de *N. crassa* [93], *Solanum tuberosum* [94] o *Arabidopsis thaliana* [95], pero a diferencia de éstas, ambos motivos de unión pueden ser funcionales. Sin embargo, la respiración dependiente de NADH externo no se estimuló con Ca^{++}, lo que sugiere que: a) la NDH-2 sensible a calcio no se expresa en este hongo, o b) el control del flujo respiratorio de esta enzima es muy bajo, por lo que la activación de la enzima no es acompañada por un incremento en la velocidad de respiración. Con respecto a la ubicación submitocondrial de las otras dos NDH-2, el análisis filogenético realizado por nuestro grupo indica que estas secuencias se agrupan en dos clados, uno de ellos con isoformas internas y el otro compuesto por isoformas externas (Figura 16).
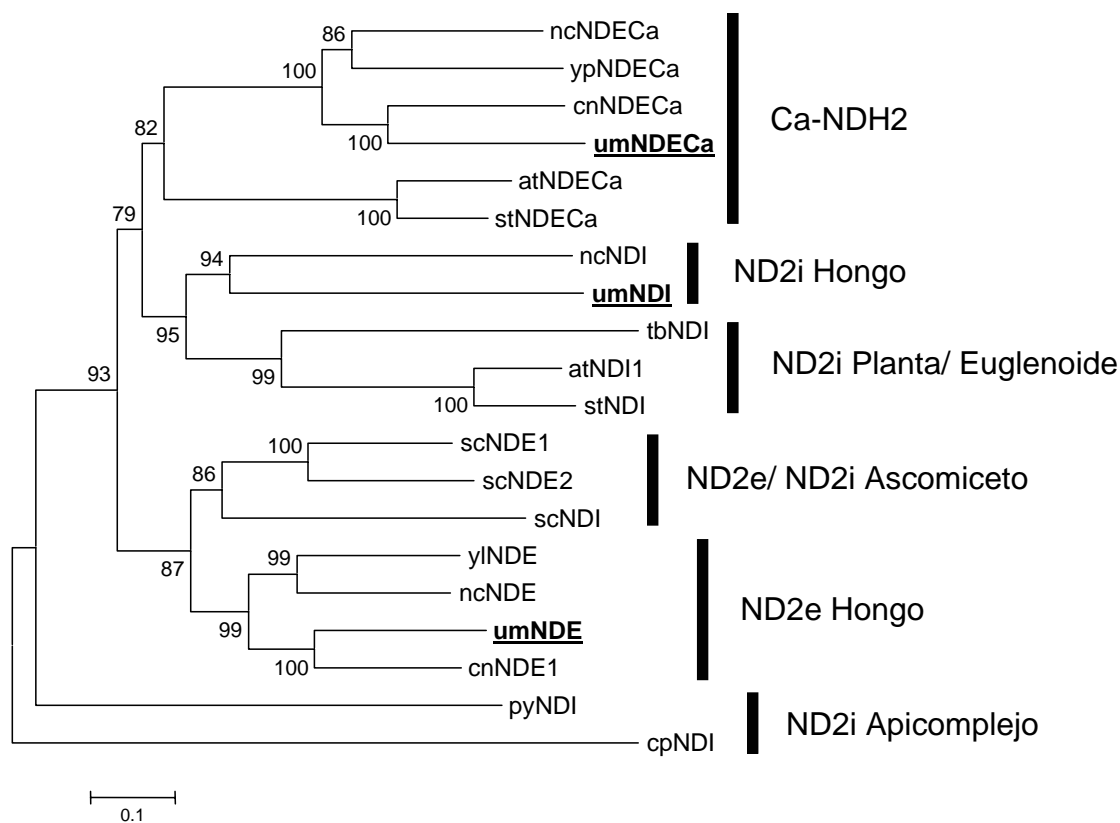

Figura 16. Árbol filogenético construido con el algoritmo de evolución mínima para las NDH-2 eucariontes. En negritas se muestra la posición de las isoformas de *U. maydis.* Las abreviaturas utilizadas son las mismas de la figura 15, y adicionalmente se incluyeron las siguientes: cn, *Cryptococcus neoformans*; py, *Plasmodium yoelli*; tb, *Trypanosoma brucei*.

Estos datos nos hacen pensar que la entidad molecular que se expresa en las condiciones evaluadas es la isoforma externa insensible a iones de calcio –umND2e- (EAK83286). Sin embargo, el análisis de los patrones de expresión de las tres iformas y experimentos de inmunodetección seran necesarios para comprobar esta hipótesis.

La identificación de las enzimas respiratorias accesorias que no bombean protones y que por lo tanto no participan en la síntesis de ATP, nos condujo a plantearnos otras dos líneas de investigación, que se centraron en el estudio de la función metabólica de la AOX y de la NDH-2 en este organismo.

4.2 La función metabólica de la AOX en *Ustilago maydis*

Para abordar este tema, se exploraron algunos de los mecanismos generales de regulación de la AOX, con la finalidad de obtener indicios sobre el papel que juega esta enzima en la fisiología celular. En particular, se probó el efecto de algunas variables de importancia ecológica sobre la actividad de la AOX y de la vía citocrómica, para entender el papel de estas vías en la adaptación metabólica de la célula.

4.2.1 Factores que modulan la actividad de la AOX

La vía citocrómica presentó una actividad constante en todas las condiciones exploradas, lo que indica que su función es fundamental para el metabolismo celular. Por otro lado, la actividad de la AOX es variable y puede ser regulada por la temperatura de cultivo, la fase de crecimiento y la fuente de nitrógeno.
El efecto de la temperatura de cultivo sobre la actividad de la AOX es difícil de explicar, debido a que este parámetro modifica una amplia variedad de procesos, como la velocidad de respiración, la tasa metabólica de la célula, la velocidad de duplicación y la propia actividad de la AOX, entre otras. Sin embargo, en la figura 17 se muestra que la temperatura produce un incremento significativo en la producción basal de H_2O_2, lo cual podría ser la señal que induce el incremento en la actividad de la AOX. No obstante, otros mecanismos regulatorios no pueden ser descartados.
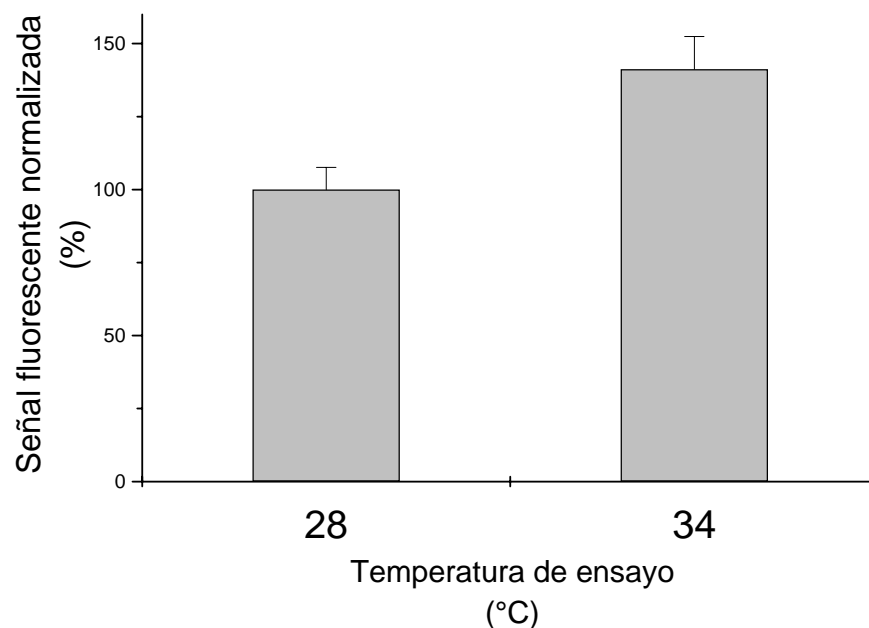
Figura 17. Efecto de la temperatura de ensayo sobre la producción de H_2O_2 en células cultivadas a 28 °C en medio YPD.

El efecto de la fase de crecimiento sobre la AOX nos pareció más fácil de entender, ya que su regulación se relaciona con los procesos de duplicación celular, de tal forma que la actividad se mantiene baja durante la fase de retraso del crecimiento y se incrementa rápidamente cuando las células entran a la fase logarítmica. En la fase estacionaria, la actividad de la AOX alcanza su máximo (4-5 veces con respecto a la actividad inicial), pero comienza a decrecer lentamente después de las 24 horas de cultivo. Este tipo de regulación sugiere que durante la fase logarítmica hay eventos que dependen de la actividad de la AOX, como podría ser la síntesis de ácidos nucléicos, que se inhibe en presencia de ROS [96], cuya producción es prevenida por la AOX.

Uno de los datos más interesantes que se obtuvieron fue que la naturaleza y cantidad de la fuente de nitrógeno puede modular la actividad de la AOX, debido a que las células cultivadas en concentraciones altas de nitrógeno en forma inorgánica (nitrato de amonio) presentan una alta sensibilidad a la inhibición por cianuro, que se va perdiendo conforme el medio de cultivo se suplementa con concentraciones crecientes de extracto de bactopeptona, lo cual sugiere que la incorporación de nitrógeno inorgánico o la síntesis de

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aminoácidos son procesos costosos, por lo que la célula evita que la AOX tenga una alta actividad en condiciones donde la economía celular se ve comprometida.

En su conjunto, los datos apuntan a que la vía citocrómica, y posiblemente los complejos respiratorios I y II, tienen una actividad fundamental, debido a que podrían constituir el sistema que produce la mayoría del ATP en este organismo, ya que su actividad está controlada de tal forma que se mantiene dentro de un intervalo restringido. Por otro lado, la AOX está sujeta a otro tipo de regulación, mucho más flexible, en la cual la célula modula su actividad en función de varios parámetros, como el estrés oxidativo, la economía celular y la fase de crecimiento, entre otras.

4.2.2 Expresión de la AOX

En la mayoría de los casos que se estudiaron, los cambios en la actividad de la AOX correlacionaron con aumentos o disminuciones en la cantidad de la proteína inmunoreactiva. Por ejemplo, las células cultivadas a 28 °C durante 24 horas y las células cultivadas a 34 °C durante 3 horas mostraron una señal pequeña en la inmunodetección y una baja actividad de AOX, con respecto a las células cultivadas durante 24 horas a 34 °C. De la misma forma, las células cultivadas en concentraciones crecientes de extracto de bactopeptona mostraron una actividad y una cantidad de AOX que tendía a incrementarse. Estos resultados sugieren que la regulación genética de la cantidad de enzima es uno de los mecanismos preponderantes para la modulación de la AOX. Sin embargo, se encontraron dos casos en los que la actividad y la cantidad de proteína no mostraron correlación, que indican que la regulación a nivel de actividad puede constituir otro mecanismo de modulación en este organismo. Por ejemplo, las células incubadas durante 40 minutos en presencia de H_2O_2 mostraron un incremento de cinco veces en la actividad de AOX, pero la concentración de proteína se mantuvo constante, lo que indica que el tratamiento con el agente oxidante incrementa la concentración de AMP o bloquea de manera parcial la actividad de la vía citocrómica, posiblemente a través de la producción de NO [97], favoreciendo la activación de la AOX. De la misma forma, las células cultivadas a 34 °C durante 8 horas presentan una actividad que es mucho menor que la que se esperaría a partir del análisis de la señal obtenida en el Western blott, posiblemente debido a que cuando las

células entran a la fase exponencial del crecimiento aumentan rápidamente la expresión de la AOX y mantienen una poza inactiva de enzima, y conforme el cultivo avanza, esta poza tiende a activarse. En otros organismos (principalmente plantas), también se han identificado fracciones inactivas de AOX, que responden de manera diferencial con respecto a la fracción activa, tanto en su velocidad de activación, como en las señales regulatorias [98, 99].

4.2.3 El impacto de la AOX sobre la economía celular

La AOX cataliza la transferencia de los electrones de la molécula de ubiquinol al oxígeno molecular, al igual que lo hace la vía citocrómica, con la diferencia de que la energía libre desprendida en la reacción catalizada por la AOX es liberada en forma de calor (~ 150 kJ/ mol) [100], mientras que la vía citocrómica utiliza una parte de esta energía (~60%) para bombear protones a través de la membrana mitocondrial, generando así un gradiente que es utilizado para sintetizar ATP. Esta propiedad le confiere a la AOX un gran potencial como enzima respiratoria de sobreflujo [101- 102], pero también introduce el problema del desperdicio de energía, por lo que para realizar un análisis completo del papel de esta enzima en el metabolismo es importante conocer el impacto que tiene en el estado energético de la célula.

Para abordar este estudio, se realizaron determinaciones del contenido de adenín nucleótidos en presencia de diversos inhibidores metabólicos, como el cianuro, el n-octilgalato (nOg), el iodoacetato (IAA) o el desacoplante pentaclorofenol (PCP), en células que presentaban diversas actividades de la AOX. Los datos más importantes son los siguientes: 1) la cantidad de ATP fue similar en células que presentaban diversas actividades de la AOX; 2) la adición de nOg no modifica la concentración intracelular de nucleótidos; 3) la adición de cianuro produce una disminución en la concentración intracelular de ATP de hasta el 50%, pero su efecto es menor en células donde la actividad de la AOX es alta; 4) la inhibición del metabolismo mitocondrial (con cianuro y nOg) no reduce totalmente la concentración de ATP intracelular y 5) en los casos donde se encontraron variaciones en la concentración de ATP, se determinó que el AMP tenía un comportamiento de la misma magnitud pero con signo contrario.

4.2.4 La contribución de la AOX a la respiración celular

Nos pareció particularmente importante que la inhibición de la AOX no tuviera efecto sobre la respiración celular y sobre la concentración de ATP, y que las células con grandes diferencias en la actividad de esta enzima presenten un contenido de ATP similar, puesto que estos datos nos brindaron un punto de partida para entender la contribución de la AOX a la respiración celular.

Para caracterizar este proceso, se formularon una serie de hipótesis sobre el funcionamiento celular al inhibir a la AOX, que cubren una gran cantidad de escenarios y pueden ser verificadas mediante técnicas estandarizadas desde hace varios años. Para facilitar su comprensión, estas hipótesis se desglosan en tres partes. El planteamiento del problema se realiza suponiendo que la mayoría de los electrones fluyen a través de la AOX, como se ilustra en el esquema 1.

Esquema 1
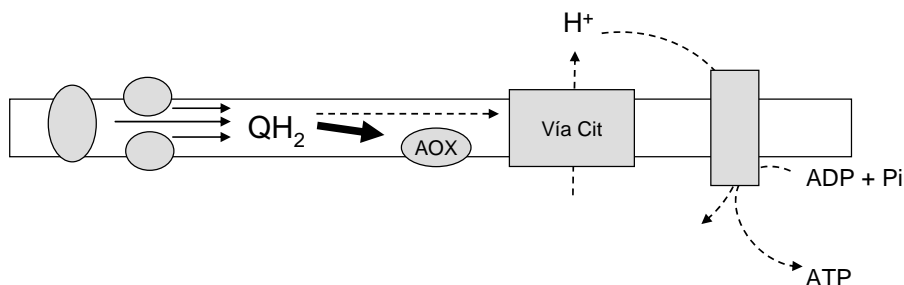
Entonces, al inhibir a la AOX, los electrones se redireccionan hacia la vía citocrómica, que bombea H^+ y que está acoplada a la síntesis de ATP, por lo que se esperaría un aumento en el potencial transmembranal y un aumento en la concentración intracelular de ATP, lo cual se ilustra en el esquema 2.

Esquema 2.

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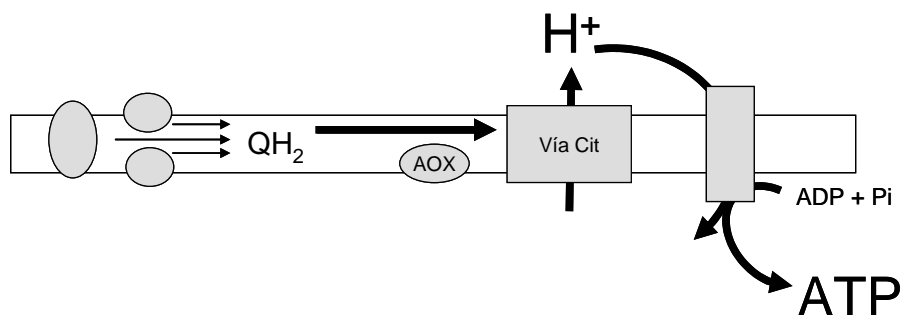
Sin embargo, el ATP podría tener un efecto negativo sobre la vía citocrómica o sobre el sistema fosforilante. De hecho, se ha reportado que el ATP es un inhibidor del complejo IV [103] y que cuando el estado energético de la célula es alto, el sistema fosforilante presenta una actividad pequeña [104, 105]. Así que al inhibir a la AOX el potencial transmembranal debería incrementarse, pero este aumento no se vería reflejado en un incremento en la concentración de ATP, sino en una disminución de la respiración celular, por el efecto negativo del ATP sobre la vía citocrómica (Esquema 3).
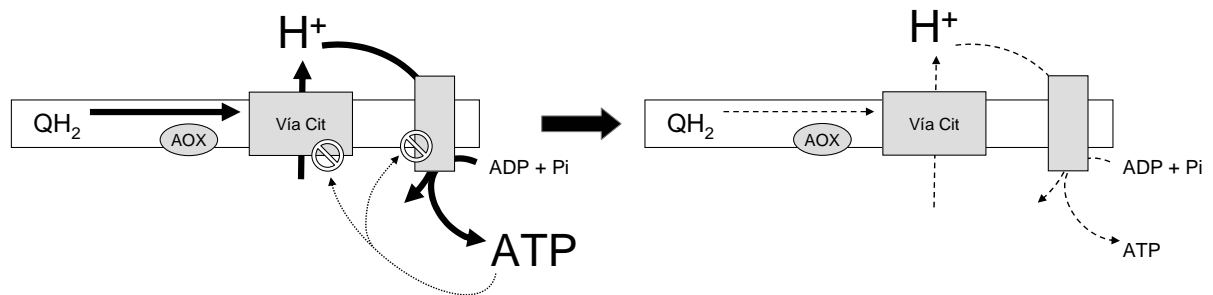
Esquema 3.

Finalmente, podría ocurrir que las células presenten mecanismos para modular la concentración de ATP, en particular aquellos que cambian la eficiencia termodinámica de la fosforilación oxidativa, como el "proton leak" o el "proton slip" [106, 107]. En este caso, el aumento en el flujo a través de la vía citocrómica y el incremento en el potencial transmembranal podría alterar la relación ATP/ O, y las modificaciones en la actividad de la vía citocrómica no serían acompañadas por cambios en la concentración de ATP, como se muestra en el esquema 4.

Esquema 4.
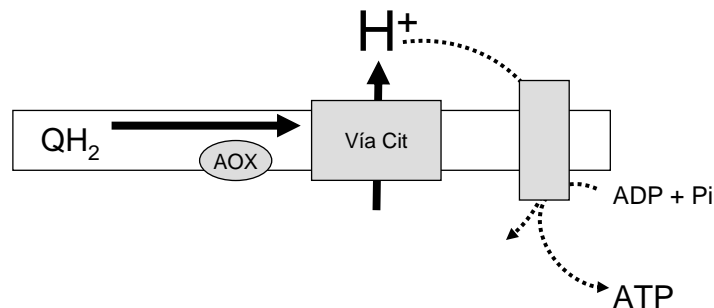
En resumen, si la AOX contribuyese en un alto porcentaje a la respiración, entonces al inhibirla se debería registrar un incremento en la concentración de ATP y en el potencial transmembranal, si la velocidad respiratoria es constante, o en otro caso, la respiración

celular se inhibiría, el potencial transmembranal debería aumentar y la concentración de ATP sería constante. Los datos experimentales mostrados en nuestro trabajo indican que la respiración celular, la concentración de ATP y el potencial transmembranal no cambian al inhibir a la AOX, lo cual indica que la contribución de esta enzima a la respiración celular es muy pequeña, como se había reportado previamente con una metodología mucho más sofisticada [108, 109]. Además, al explorar el efecto de la temperatura de ensayo sobre la actividad de la AOX, se descubrió una prueba independiente que corrobora la validez del método, la cual consta de la inhibición casi completa, pero transitoria, de la respiración celular al adicionar cianuro, lo que indica que antes de la adición del cianuro la AOX tenía una actividad muy pequeña, y que se requiere de cierto tiempo (varios segundos) para que el metabolismo se reajuste y se active a esta enzima.

4.2.5 La plasticidad metabólica celular y la AOX

El efecto del cianuro sobre los adenín nucleótidos nos permitió elaborar una de las conclusiones más importantes del trabajo. Como se mencionó, el cianuro disminuye hasta en un 50% la concentración de ATP intracelular, pero su efecto es menor en células con alta actividad de AOX. Además, se había mostrado que esta enzima es capaz de sostener tanto la actividad respiratoria, como la producción y mantenimiento de un potencial transmembranal en la mitocondria cuando la vía citocrómica se inhibe. Como se puede observar, la presencia de la AOX incrementa la plasticidad metabólica de la célula, permitiendo que los procesos que sintetizan ATP, como el ciclo de Krebs, la glucólisis, el complejo I y la ATP sintetasa sigan activos a pesar de que la vía citocrómica ha sido inhibida, y adicionalmente mantiene activas otras vías aerobias, lo que evita que el metabolismo se torne fermentativo. Es importante recalcar que la fermentación es un proceso costoso, ya que se producen y expulsan metabolitos carbonados en estados de oxidación incompletos. Ahora bien, los organismos de vida libre se encuentran expuestos a diversas situaciones, que surgen de las interacciones con otros organismos o bien con el medio ambiente, capaces de producir la inhibición de los complejos III o IV, como son la producción de NO en la respuesta de diversos organismos contra la invasión de patógenos [110], la cianogénesis de las plantas y de algunos microorganismos [111, 112], la

producción de antagonistas fenólicos que inhiben al complejo III (antimicina o metoxiacrilatos) por bacterias del suelo [113-117], y otros factores medio ambientales [118-126] como un bajo pH, la temperatura, la baja disponibilidad de Cu^{++} o la concentración de iones como el S^{2-}. Tomando en cuenta estas circunstancias, es evidente que la presencia de una oxidasa terminal accesoria inducible sería benéfica para los organismos de vida libre.

4.2.6 La regulación de la glucólisis

En nuestro trabajo se observó que el IAA, el cual es un inhibidor de la gliceraldehído 3-fosfato deshidrogenasa [127], no tiene un efecto importante en la concentración de ATP, a menos que se encuentre en presencia de nOg y cianuro, es decir, cuando el metabolismo mitocondrial es inactivo. Esto sugiere que la glucólisis podría tener una participación importante en la síntesis de ATP, particularmente cuando la función mitocondrial es limitada, el cual es un proceso conocido como efecto Pasteur, e indica que la fosfofructocinasa I podría ser un punto importante de control en la glucólisis en este hongo, puesto que esta enzima coordina la transición aeróbica de esta vía [128]. De hecho, los experimentos realizados por Guerra y Pardo (datos no publicados) confirman esta propuesta.

4.2.7 Regulación de la AOX en los hongos y protistas

Se observó que las condiciones que promovieron una disminución en la concentración de ATP ocasionaron un incremento en el contenido de AMP, lo que sugiere que en *U. maydis* el sistema amortiguador de adenilatos, compuesto por la adenilato cinasa [129, 130], es activo. Nos pareció importante este efecto, debido a que se ha descrito ampliamente que las moléculas mensajeras que llevan la información sobre el estado energético de la célula son el ATP y el AMP [104].

La entrada a un estado energético bajo tiene como consecuencia que la concentración de AMP aumente, como sucede al inhibir a la vía citocrómica. Este metabolito desata un proceso de rescate metabólico, a través de la activación tanto de la glucólisis [128] como de la AOX [74, 75], con lo cual se produce una respuesta para mantener activos al ciclo de

Krebs, a la glucólisis aerobia y a la síntesis de ATP mitocondrial, evitando la disminución abrupta en el contenido de ATP y manteniendo activos diversos procesos biosintéticos, particularmente los que dependen del ciclo de Krebs.

La activación por AMP podría ser el mecanismo por el cual la AOX pasa de ser una vía de sobreflujo (que sólo toma una pequeña proporción de los electrones) [101, 102], a una vía de flujo, en donde una gran proporción de los electrones fluyen a través de ella. Esta regulación contrasta con la reportada para las plantas, en donde se sabe que en estados energéticos altos la vía citocrómica disminuye su actividad, debido a la restricción por adenilatos [103, 104], es decir, por una baja disponibilidad de ADP, lo cual produce la acumulación de metabolitos como el NADH, el piruvato, el isocitrato y el malato, entre otros. El incremento en la concentración de malato e isocitrato produce a su vez un incremento en la concentración de NADPH, glutatión reducido y tiorredoxina, que activan a la AOX, permitiendo que el metabolismo mitocondrial no se detenga, y de hecho la fotorrespiración depende de la actividad de la AOX [101, 102].

4.2.8 La AOX como mecanismo que previene la formación de ROS

En el ciclo catalítico de los complejos respiratorios I y III se lleva a cabo la producción de una molécula del ión semiquinona, que es fundamental para el funcionamiento del ciclo Q y para el bombeo de protones por estos complejos [44, 45]. Sin embargo, la semiquinona puede reaccionar con el oxígeno molecular, produciendo el radical anión superóxido, que inicia la cascada de producción de otras ROS [23]. De hecho, la mayor parte de la producción de estos intermediarios es el resultado del metabolismo mitocondrial [131-134] y se encuentra bien descrito que los procesos que alteran la función respiratoria modifican la producción de ROS [133], como el envenenamiento con inhibidores, la hiperreducción de la poza de ubiquinona, la concentración y el tipo de sustratos respiratorios, el grado de acoplamiento de la mitocondria, etc.

Por otro lado, la célula cuenta con varias líneas de protección contra el estrés oxidativo que previenen la producción de ROS (en particular del ión superóxido), que eliminan a las especies reactivas, o que reparan el daño que éstas producen. Dentro de los procesos mejor caracterizados que previenen la producción de ROS se encuentra la AOX [134]. El mecanismo de protección está vinculado al mecanismo catalítico de esta enzima, en el que,

a diferencia del complejo III, no se produce la molécula de semiquinona a pesar de que la transferencia de los electrones al oxígeno es univalente, posiblemente porque los dos electrones del ubiquinol son transferidos en un solo paso al centro Fe-Fe, para posteriormente ser transferidos (de uno en uno) hacia el oxígeno [135]. Además, la AOX evita la producción de las especies reactivas al competir por sustrato con la vía citocrómica y al mantener en un estado relativamente oxidado a la poza de ubiquinol.

En *U. maydis*, al igual que en muchos otros sistemas [122, 134, 136-139], se determinó que la AOX forma parte de la maquinaria para combatir el estrés oxidativo, ya que la producción de H_2O_2 se incrementa de manera importante al inhibir a la AOX. De hecho, en términos cuantitativos la inhibición de esta enzima genera aún más ROS que la inhibición de la vía citocrómica. Otro de los resultados que apoyan esta función es el incremento en su actividad al incubar a las células en presencia de H_2O_2.

4.2.9 Una buena inversión

Dadas las propiedades que se han mencionado, la relevancia de la AOX para una gran cantidad de organismos puede ser entendida en términos de la relación costo/ beneficio. Los datos apuntan a que la AOX genera bajos costos, ya que a pesar de que presenta un gran potencial como enzima disipadora de energía, se encuentra estrictamente regulada y sólo se activa en ciertas condiciones, y por otro lado tiene una baja participación en la respiración, por lo que un número limitado de electrones fluyen a través de esta vía y por lo tanto la energía desperdiciada es mínima. Además, esta enzima produce altos beneficios, puesto que su actividad es fundamental para el control de la producción de las ROS y adicionalmente su presencia evita que el metabolismo energético e intermediario se colapse cuando la vía de los citocromos se encuentra inhibida, una condición que puede ser generada por diversos factores naturales. Como se puede apreciar, la adquisición de la AOX ha resultado una buena inversión para los organismos aerobios de vida libre.

4.3 La función metabólica de la NDH-2 externa en *Ustilago maydis*

La NDH-2 cataliza una reacción redox similar a la del complejo I, pero en contraste con éste la NDH-2 no es capaz de mover unidades de carga a través de la membrana

mitocondrial interna [59], lo que en primera instancia parecería que podría generar una presión de selección negativa para las células que la presentan. Sin embargo, dada su amplia distribución en arqueobacterias, eubaterias y eucariontes, en organismos con un metabolismo de tipo fermentativo, en aerobios, en anaerobios, y en fotosintéticos o en quimiolitótrofos [59, 140], así como en organismos de vida libre o parásitos, es posible que su actividad sea importante en todos ellos, aunque su relevancia y función podría variar en cada caso.

La NDH-2 tiene otras grandes diferencias estructurales con el complejo I, que la hacen una enzima mucho más sencilla. De entrada, su masa molecular puede ser 1/20 o 1/30 de la masa molecular del complejo I, está codificada por un gen y tiene un solo cofactor (FAD o FMN) [59], lo que contrasta con la multiplicidad de subunidades y cofactores del complejo I [24, 27, 30]. Además, es una enzima monotópica sin segmentos transmembranales, que aparentemente no requiere de una maquinaria especializada para unirse a la membrana [94, 141].

Los eucariontes presentan tres isoformas de la NDH-2 que se clasifican de acuerdo a su ubicación en los compartimentos mitocondriales o por la activación por calcio [59]. Las tres isoformas se encuentran unidas a la membrana interna mitocondrial, pero su disposición es diferente, de tal forma que la isoforma interna se encuentra orientada hacia la matriz mitocondrial, mientras que las dos isoformas externas se orientan hacia el espacio intermembranal, y se diferencian entre sí debido a que una de ellas presenta un segmento con dos motivos de unión a calcio, de tipo mano EF. La función metabólica de cada una de las isoformas no ha sido estudiada con profundidad, pero se han propuesto ciertas funciones para algunas de ellas. Con respecto a la isoforma interna, se ha descrito que su simplicidad estructural es aprovechada por algunos organismos, especialmente en condiciones donde es necesaria una respuesta rápida para ajustar el metabolismo [142, 143], ya que esta enzima se puede plegar rápidamente y su expresión depende de la regulación de un solo gen, en comparación con la expresión y ensamblaje del complejo I, que presenta muchos cofactores y diversos niveles de regulación. Por otro lado, se ha reportado que las isoformas externas forman parte de la maquinaria mitocondrial requerida para oxidar al NADH citosólico, y de hecho en *S. cerevisiae* éste es el mecanismo de tipo lanzadera más importante [144, 145], aunque se encuentra acompañado por otros sistemas.

Dada la ubicación de la NDH-2 en *U. maydis*, ésta podría tener la misma función que la descrita para su homóloga en *S. cervisiae*. Sin embargo, la búsqueda informática en el genoma de nuestro modelo de estudio reveló que este organismo tiene el complemento genético completo para todas las lanzaderas descritas, como la lanzadera de aspartato-malato, la de malato- oxaloacetato, la de acetaldehído etanol y la de glicerol 3-fosfato [144], por lo que se realizaron experimentos para establecer cuáles de estas son activas y para conocer la importancia relativa de la NDH-2.

Se determinó que la actividad de la NDH-2 no muestra grandes diferencias al cultivar a las células en diversas condiciones de cultivo. De hecho, los experimentos donde se encontraron variaciones en la actividad de la AOX (variando la temperatura y la cantidad y naturaleza de la fuente de nitrógeno y carbono) fueron originalmente diseñados para encontrar cambios en la actividad de la NDH-2. Este comportamiento es similar al mostrado por la vía citocrómica y sugiere que la actividad de esta enzima es fundamental en las diversas condiciones metabólicas ensayadas. Por otro lado, la lanzadera de glicerol 3-fosfato mostró una actividad moderada y las demás lanzaderas presentaron actividades muy pequeñas, y en algunos casos despreciables, lo que apunta a que la NDH-2 es el sistema más importante de oxidación del NADH externo.

Para evidenciar que la lanzadera de acetaldehído- etanol y la de malato- oxaloacetato pueden ser activas, se decidió cultivar a las células en presencia de etanol o malato. En las células cultivadas con etanol, la NDH-2 también presentó una buena actividad y se pudo apreciar que las isoformas citosólica y mitocondrial de la alcohol deshidrogenasa son activas, lo que apunta a que la lanzadera de acetaldehído- etanol podría ser funcional. Sin embargo, este hongo presenta una acetaldehído deshidrogenasa mitocondrial que podría tomar el acetaldehído generado en la matriz, canalizando los esqueletos carbonados hacia el ciclo de Krebs, evitando así su efecto nocivo, pero también su salida al citosol, lo que impediría el funcionamiento de la lanzadera. Dentro de los experimentos que son necesarios para aclarar este punto están la determinación de la actividad de la alcohol deshidrogenasa y acetaldehído deshidrogenasa en fracciones citosólicas y mitocondriales, y la cuantificación de la producción de acetaldehído en el sistema de células permeabilizadas o en mitocondrias aisladas, para comprobar si este intermediario puede atravesar la membrana mitocondrial interna.

En las células cultivadas en presencia de malato se determinó que el consumo mitocondrial de este intermediario es muy activo (comparable al obtenido con NADH externo), lo cual es una de las condiciones para que la lanzadera de malato- oxaloacetato pueda operar. La otra condición es que el oxaloacetato generado en el interior de la mitocondria pueda salir al citosol, lo que de hecho parece ocurrir, ya que la respiración con NADH externo y succinato se inhibe en presencia de rotenona, la cual no inhibe al consumo de oxígeno en presencia de NADH externo o succinato por separado. Estos resultados indican que los electrones del NADH externo son introducidos a la matriz mitocondrial, para ser tomados por el complejo I. El mecanismo subyacente de este proceso podría ser parecido al representado en la figura 17, en donde el succinato es tomado por al ciclo de Krebs y convertido en oxaloacetato, para posteriormente salir de la mitocondria y ser reducido hacia malato, y finalmente ser oxidado intramitocondrialmente.
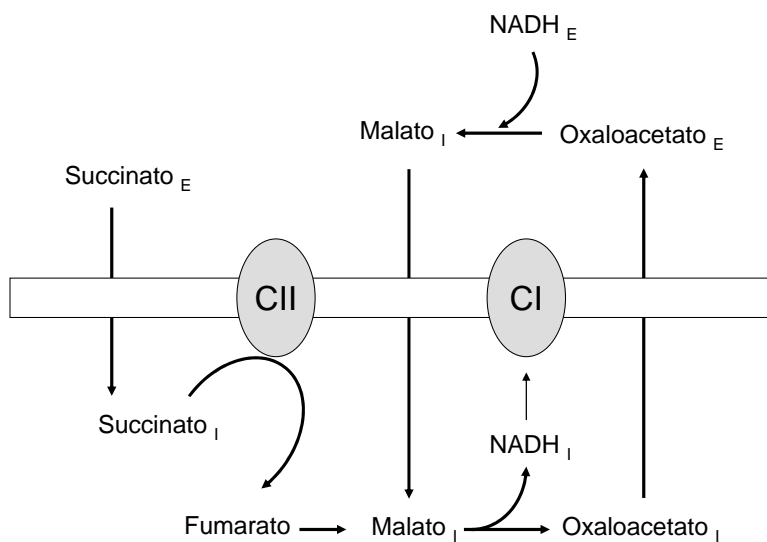
Figura 17. Mecanismo hipotético para explicar la inhibición por rotenona de la respiración obtenida en succinato y NADH externo.

Para comprobar esta propuesta se requieren realizar experimentos para determinar si el oxaloacetato efectivamente se exporta y si su velocidad de transporte puede ser afectada por otros sustratos del ciclo de Krebs, particularmente por el piruvato. Además, sería interesante conocer si el consumo de malato depende exclusivamente de la actividad de la

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malato deshidrogenasa mitocondrial, o si la enzima málica participa en este proceso. También sería importante aclarar si la malato deshidrogenasa interviene en algún proceso de canalización enzimática, particularmente con el acarreador de oxaloacetato.

Finalmente, para entender cuál de los sistemas de lanzadera es el más importante, se requeriría evaluar la Km aparente por NADH de cada una de las lanzaderas, así como la concentración intracelular de los piridin nucleótidos.

5. CONCLUSIÓN

"Yo no soy nadie:
Un hombre con un grito de estopa en la garganta y una gota de asfalto en la retina".

"Mios son el pecado y la caida.
Y esas lágrimas
y esa baba epiléptica
y esas gotas de angustia
y esas manchas de sangre sobre el suelo,
como monedas escapadas de la bolsa rasgada de mi cuerpo,
están ahí para pagar mis deudas...
unas deudas antiguas y unos réditos"
León Felipe, 1943

La cadena respiratoria mitocondrial del hongo fitopatógeno *Ustilago maydis* consta de los complejos respiratorios I-IV, que realizan la transferencia escalonada de los electrones del NADH y del succinato hasta el oxígeno molecular, en un proceso acoplado al bombeo de protones a través de la membrana interna mitocondrial. Este organismo también presenta dos componentes respiratorios accesorios, la oxidasa alterna (AOX) y a la isoforma externa de la NADH deshidrogenasa alterna (ND2e), las cuales, en contraste con el complejo I y con la vía citocrómica, no son capaces de bombear iones, por lo que no participan en la síntesis de ATP, y de hecho, tienen una contribución negativa al estado energético de la célula.

A pesar de esta característica, ambas enzimas presentan una amplia distribución, abarcando una gran variedad de linajes taxonómicos, lo que hace suponer que juegan papeles preponderantes en el metabolismo celular. Desafortunadamente, con excepción de plantas y hongos ascomicetos, existen pocos reportes donde el papel de estas enzimas haya sido explorado con profundidad. De hecho, *U. maydis* es el primer basidiomiceto donde este tipo de investigaciones se han realizado, lo cual tiene especial importancia debido a que se ha descrito que los componentes respiratorios accesorios le brindan resistencia hacia plaguicidas comerciales, especialmente a los que inhiben al complejo I y III, como el piridaben y algunos derivados de los metoxiacrilatos, como la estrobilurina.

Los estudios realizados en este trabajo indican que la AOX juega dos papeles fundamentales en *U. maydis*, forma parte de los mecanismos que previenen la producción de ROS y le permite a la célula adaptarse a condiciones generadas por factores externos que limitan o inhiben la actividad de la vía citocrómica. Por otro lado, presenta una serie de características que permiten que su función se lleve a cabo sin afectar de manera significativa la economía celular. Por ejemplo, su actividad está estrictamente regulada a diversos niveles, ya que la célula controla tanto su concentración como su actividad, de tal forma que sólo es activa en condiciones puntuales. Además, en condiciones regulares no participa de manera importante en la respiración celular.

Se ha descrito que *U. maydis* es un organismo aerobio obligado, de tipo no fermentativo, lo que indica que la mayoría del NADH que se produce por la glucólisis, o por otras vías, es oxidado en la mitocondria. Dada su ubicación submitocondrial, la función evidente de la ND2e es la oxidación del NADH citosólico, formando parte de los sitemas de lanzadera

redox mitocondriales. Sin embargo, *U. maydis* tiene el complemento genético para todas las lanzaderas descritas, por lo que se realizó una exploración minuciosa de los sistemas mitocondriales de oxidación del NADH citosólico que son activos en este organismo. Se demostró que la ND2e podría ser el sistema de lanzadera más importante en este hongo, ya que presenta una actividad constitutiva, que en muchos casos fue considerablemente mayor que la del resto de las lanzaderas. La ND2e puede estar acompañada por otras rutas, como la lanzadera de glicerol 3-fosfato, la de malato- oxaloacetato o la de acetaldehído etanol, las cuales aparentemente tienen una regulación flexible, que depende de las condiciones metabólicas de la célula.

Memento mori.

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